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# INFLUENCES OF LEGUMES ON THE REPRODUCTIVE PHYSIOLOGY OF MICE

WENDELL WILLIAM LEAVITT

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INFLUENCES OF LEGUMES ON THE  
REPRODUCTIVE PHYSIOLOGY OF MICE

BY

WENDELL WILLIAM LEAVITT

A. B., Dartmouth College, 1959

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This thesis has been examined and approved

Paul A. Wright

W. A. Keener

George M. Moore

Philip J. Sawyer

David H. Loubser

May 23, 1963

Date

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## I. INTRODUCTION

A great deal of progress has been made in understanding normal reproductive processes, and valuable information has been obtained concerning reproductive disorders. A recent estimate by Hansel (1961), based on the improvement between 1950 and 1961 in the conception rates of dairy cattle bred artificially in New York, indicates an annual saving of some 57 million dollars, if applied to the nation as a whole. The perpetuation of the dairy industry in the Northeast, competitive with other areas in which costs of production are lower, depends on increased animal productivity. Efficient reproduction is one of the most essential factors in this improvement. While increases in such efficiency have been made in recent years, particularly with artificial insemination, much more remains to be done before the present average rate of 1.6-2.0 services per conception is reduced to an ideal of one.

The inefficiency encountered in the control of dairy cattle reproduction stems largely from an incomplete understanding of the hormonal control of reproductive processes. It is known that many reproductive disorders are caused by aberrant function of endocrine glands. These can result from endogenous or exogenous factors. A large effort has gone into the characterization of the intrinsic causes of reproductive disfunction. However, much less information

has been obtained on the influence of extrinsic factors on reproductive physiology. Although breeding performance is affected by such elements as season of mating, temperature, light, social impact, pathological organisms, etc., one of the most important (and most often underestimated) factors is the diet.

Information derived from primitive peoples, from the literature of "popular" medicine, from publications on medical botany, and from old materia medica concerning the use of herbs and various plant materials for fertility control purposes has been appearing for many years. The validity of these accounts is to some extent held in question by modern investigators. However, confidence in such sources of information is strengthened when it is recognized that many valuable drugs have been isolated from plants used by primitive people for generations (de Laszlo and Henshaw, 1954).

Recent research on minor forage constituents has revealed that they often can cause unusual physiological responses in animals. Many unidentified factors have been reported in forages, but only a few, such as folic and thioctic acid, have been isolated in pure form. Included among the still unidentified factors are: a growth factor for chicks, turkeys and guinea pigs, found in fresh-forage juice ("grass juice factor"); an uncharacterized water-soluble factor in alfalfa which improves utilization of vitamin A; a substance beneficial to the survival of thyrotoxic rats; a factor which potentiates the symptoms of

hypervitaminosis A. Also uncharacterized are: a growth factor for baby pigs; materials which improve egg production; a lactation-stimulating factor; substances capable of counteracting toxicity due to glucoascorbic acid, to massive doses of  $\alpha$ -estradiol, to mineral oil, to fish oil, to Tween-60, or to other nonionic surface-active agents. Evidence likewise exists on factors essential to normal reproduction of swine, rabbits, and sheep (Kohler and Bickoff, 1961).

There are reports of many more forage components whose identities and activities in animals remain unknown. The United States Department of Agriculture, Agricultural Research Service, is interested in research on the identification and detailed study of forage components. Special emphasis has been given to the following: (1) constituents causing increased growth rates; (2) factors stimulating milk production; (3) materials preventing disease; (4) factors inhibiting growth; (5) components inhibiting or potentiating certain physiologic responses.

Reports of breeding problems with dairy cattle which cannot be explained by known pathological organisms or by systemic disorders have been appearing for some time throughout the northeast region of the United States. Ladino clover and other legumes have been implicated in these disorders. Heinzelmann (1960) cited an example of this in an experiment conducted at the University of New Hampshire dealing with the effect of trace mineral deficiencies in cattle resulting from heavy fertilization of the soil. It was noted that animals consuming Ladino clover-bromegrass hay had a signif-

icantly lower number of first-service conceptions than those receiving timothy hay. The same author presented the results obtained from feeding low levels of diethylstilbestrol and Ladino clover silage to dairy heifers. It was concluded that the low levels of estrogenic activity found in the rations used in this experiment had no apparent adverse effect on the reproductive performance of dairy heifers.

Because of the nature of the problem and the high cost of working with dairy cattle, subsequent work by Wright (1960) was carried out with rabbits. Complete infertility resulted when Ladino was fed both prior to and during theoretical gestation periods. Although the mechanism was not pinpointed, failure of ovulation and implantation was observed. The material presented in this dissertation is a result of a continuation of these studies. However, mice were used as experimental animals both for comparative purposes and for further evaluation of the mechanisms involved. This work was supported in part by regional research funds of the Agricultural Experiment Station, under the NE41 project, Endocrine factors affecting reproduction in the bovine female. The specific area of the project comes under the subdivision, Environmental factors acting through the endocrine system which affect reproduction.



## II. REVIEW OF LITERATURE

### 1. GENERAL

Diet and reproduction have been of interest to mankind for centuries. Man has been concerned with determining the proper nutritional requirements for more efficient reproduction for at least 5000 years. We are uncertain about events prior to recorded history, but folklore passed on from ancient peoples leads one to suspect that this problem has been of concern since the beginning of the human race. A review of the literature provides one with a multitude of data pertaining to many aspects of reproduction. A host of reports are concerned with the physiology and endocrinology of reproductive phenomena. Others are directed at determining various nutritive requirements for different phases of reproduction. Fewer data are present, however, on specific dietary regimens and their effects on reproductive capacity.

With this in mind, an attempt has been made to review literature related to the subject of this dissertation. In most cases, specific information on feeding legumes does not apply to laboratory mice. However, many results with other animals have been obtained, and those that are pertinent have been included.

An attempt by Russell (1948) was made to consolidate data on the effects of diet on the ability of laboratory animals to produce and rear their young, and to assess the

minimum daily intake of essential nutrients consistent with satisfactory reproductive performance. Although most of the material reviewed concerns rats, many facts relating to mice are included. Morris (1944) reviewed the nutritive requirements of normal mice for growth, maintenance, reproduction and lactation. In inbred strains of mice fed synthetic diets, Fenton and Cowgill (1947) found that vitamins A, D, E, K, B<sub>1</sub>, B<sub>2</sub>, nicotinic acid, pantothenic acid, pyridoxine, choline, inositol, p-aminobenzoic acid, folic acid, biotin, and cystine were all required for reproduction and lactation. Brown et al. (1960) presented a general discussion of the influence of nutrition on reproduction in laboratory rodents.

Snell (1941) has a chapter on reproduction of the laboratory mouse, including a comprehensive bibliography. Much of the basic endocrinology of this animal is covered by this author. For raising laboratory mice, Eaton and Cabell (1949) gave an effective account of the procedure. The Committee on Standards of the Institute of Laboratory Animal Resources (1957) proposed elaborate rules and regulations for the housing and management of laboratory mice. Miller and Wood (1961) offered an outline for an economical laboratory mouse colony differing somewhat from commonly-accepted procedures.

The relation of litter size to litter order was recently studied by Biggers, Finn and McLaren (1962). In normal female mice and mice with one ovary removed, there was an initial increase in litter size, followed by a

plateau period. Then a period of linear decline occurred in which the number per litter decreased by one in each successive gestation. A survey of previous data on long-term reproductive performance in polytocous mammals showed that this pattern is the usual one in rats and mice.

Basic endocrine mechanisms controlling different phases of reproduction are for the most part only generally understood. The details of the site and mechanism of hormone action, the qualitative and quantitative relationship between sex steroids, and their precise role in the hypothalamo-pituitary-gonadal axis are not well-known (Erb and Gomes, 1962).

Three separate gonadotropins are generally recognized as originating from the anterior pituitary gland. These are luteotropin (LTH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). It is well-known that maturation of ovarian follicles is dependent on secretion of gonadotropin. FSH brings about enlargement of follicles, but these are unable to secrete estrogen unless small amounts of LH are present. Until recently, the feed-back mechanism regulating FSH release was not well understood, and it has been especially difficult to establish separate systems for LH and FSH (Erb and Gomes, 1962). Turner (1960) noted that FSH and LH release are now thought to be controlled by the levels of estrogen and progesterone in the circulation. Ovulation occurs while blood LH levels are still increasing, and there is an immediate decline in circulating estrogen following this event. The corpus luteum becomes functional under

LTH stimulation. The major hypothalamic effect of LTH in the rat is one of inhibition, so that when inhibition is removed, corpus luteum maintenance is prolonged (Rothchild, 1960a). Progesterone has an LTH-sustaining effect (Rothchild, 1960b) and oxytocin, along with other hypothalamic products, inhibits LTH secretion (Faulkner and Hansel, 1962). It is evident that control between gonads and anterior pituitary is via the hypothalamus (Harris, 1961), and it appears that the uterus is involved in some ill-defined manner (Duncan et al., 1961).

In electron micrographs, Farquhar and Rinehart (1954, 1955) have been able to identify two kinds of acidophils and four kinds of basophils in the anterior pituitary of the rat. Thyrotrophs (beta cells) and gonadotrophs (delta cells) can be distinguished on the basis of differences in form, position, and staining properties. Delta cells are rounded and occur mainly in the peripheral parts of the gland usually situated along blood vessels. Both cells are PAS positive, while only beta cells stain with aldehyde-fuchsin. Purves and Griesbach (1952) described two types of delta cells in the rat. These were differentiated by their response following manipulation of the reproductive cycle. One type is located at the periphery of the gland, and the other is more centrally distributed. These authors suggested that the peripheral type may produce FSH and the central type LH. Farquhar and Rinehart (1954) agreed with this interpretation. The latter authors were also able to distinguish lactogenic (LTH) acidophils from growth-hormone

acidophils. LTH-producing cells have dense secretory granules which are larger than those in other acidophils.

The ovary can produce progestogens, androgens, estrogens, and a nonsteroid hormone, relaxin. As earlier reviewed by Csapo (1961), Emmens (1959), and Stormshak and Erb (1961), progestogen originating in the corpus luteum is essential for pregnancy maintenance in several animals, including the mouse. The follicular structure producing estrogen has never been definitely determined (Turner, 1960). This hormone is also produced by the corpus luteum along with progesterone (Erb and Gomes, 1962).

Velle (1961) recently reviewed the literature on estrogens in domestic animals. No endogenous estrogen seems to have been unequivocally identified in the peripheral blood of any species (Engel and Cameron, 1959). Even though estradiol  $17\beta$  and estrone are believed to be the major secretory products of the ovary in the human, pig, cow, horse and certain others, Engel (1959) noted that in the rat and mouse it is not known whether these compounds are the primary estrogenic secretions.

A review of nutrition-endocrine interrelationships with special emphasis devoted to the anterior pituitary was presented by Ershoff (1952). It was emphasized that function of the anterior pituitary and the target organs is dependent on the nutritional state and composition of the diet. Conversely, the various hormones of the anterior pituitary and its target organs profoundly affect absorption, utilization and excretion of nutrients and requirements of

the organism for specific dietary factors. Nutritional factors can be extremely important conditioning agents. Visscher, King and Lee (1952) noted that the onset of puberty in mice is delayed by reducing the caloric intake. However, these animals lived longer than well-fed controls, and females developed full reproductive competence after being returned to a complete caloric intake.

The effects of caloric restriction are especially noticeable in respect to gonadotropic secretion. Rinaldini (1949) fed young albino rats on low-calorie diets and noted a weight loss of about 35%, regressive changes in ovaries, and interruption of estrous cycles. It was concluded, after an assay of the gonadotropin content of pituitary glands, that the anterior lobe stored these products during inanition and did not release them into general circulation. More recently, Srebnik, Nelson and Simpson (1958, 1961) and Srebnik and Nelson (1962) have shown that the absence or low levels of dietary protein in rats resulted in a marked atrophy of the ovaries and accessory organs with an early loss of estrous cycles. It appeared that protein deprivation explained the failure of gonadotropin release. Females had as much LH and slightly more FSH in the anterior pituitary as normally-fed animals. In contrast, males showed a decreased hypophysial gonadotropic content as well as retarded release of tropins.

It is becoming increasingly apparent that the nutrition of an organism is dependent on considerably more than its intake of food. Ershoff (1952) listed many factors

which result in increased requirements beyond the usual or average range. Some examples are certain environmental conditions, pregnancy, and lactation. The requirements for a number of nutrients are also increased under conditions of stress. McClure (1958) noted that previous data suggested temporary nutritional stress at or about the time of mating could cause infertility. Using mice, he was able to show that three 48-hour periods of fast separated by two 48-hour periods of normal feeding resulted in complete infertility. However, it was found that the residual effect of starvation was short, with fertile matings occurring 10 to 16 days later.

Lutwak-Mann (1958) presented material on the relation of vitamins and other nutritional factors to gonadal function. Mentz and Odenal (1950) discussed the role of the B vitamins, lysine, and tryptophan in the metabolic fate of estrogens. The B-complex vitamins are essential for the inactivation of estrogens in the liver. Androgens are less susceptible. Among the nutrients known to be concerned with the function of the adrenal cortex, ascorbic acid, pantothenic acid, and sodium have been most thoroughly investigated. Eisenstein (1957) discussed these in connection with the production of adrenal steroids.

Sachs (1962) analyzed the regulation principles in steroid hormone production. The co-factors necessary for biogenesis of steroid hormones are provided by reactions of carbohydrate metabolism. Acetyl CoA,  $\text{NADH}_2$ , and ATP are produced in the course of glycolysis.  $\text{NADPH}_2$  results from

the hexosemonophosphate shunt. The synthesis of corticosteroids, progestogens, androgens, and estrogens is described starting from glucose, acetyl-CoA, cholesterol, and pregnenolone. It is obvious that a nutritive disorder which produces an imbalance or deficiency of the co-factors and substrates involved in steroid biogenesis would undoubtedly result in faulty hormone synthesis.



## 2. FORAGE FACTORS AFFECTING THE REPRODUCTIVE SYSTEM

A great variety of plants constitute the staple foods of domestic animals which are the main providers of meat, milk and other products for man. Much data has accumulated to indicate that many types of green forage contain unidentified factors with physiological activity. Friedman and Friedman (1934) reported a gonad-stimulating substance in extracts of alfalfa. Later, the same authors (1939) reported similar activity from other sources and observed a great deal of variability between different lots of test material (Friedman and Mitchell, 1941). Gonadotropic effects of juices from cereal grasses were studied using a rabbit ovulation test (Bradbury and Hodgson, 1942; Borasky and Bradbury, 1942). The ability to induce formation of corpora lutea by extracts injected into estrous rabbits was used as the criterion of evaluation. Bradbury (1944) pointed out that the effective principle in plants was not primarily gonadotropic, but rather neurotoxic. He also noted that extracts having effect in rabbits were inactive in rats and mice.

Bodenheimer and Sulman (1946) observed that factors in young grass may affect the endocrine system in such a way as to have a strong influence on productivity as well as on reproduction. An unidentified factor in oats was found to produce early vaginal opening and to stimulate

ovarian activity in immature rats (Gomez, Hartman and Dryden, 1941). This material ("sex maturity factor") was orally active, water soluble, and could be concentrated by alcohol precipitation of the grass juice. Evans and Evans (1949) reported that the onset of sexual maturity was markedly hastened in mice receiving a supplement of either dried or fresh grass in their diet. Growth curves of animals on control and test diets did not differ appreciably. Ershoff, Hernandez and Matthews (1956) noted that the deleterious effects of estrogen feeding on ovarian development in the immature rat could be counteracted by the concurrent feeding of dried alfalfa. They suggested that the protective action of alfalfa might be due to an orally effective gonadotropin or possibly to an estrogen inhibitor.

The effects of various plant preparations on the fertility of laboratory mammals has been the subject of many investigations (East, 1955 a, b, c; Kendall, Salisbury and Van Demark, 1950; Wright, 1960). However, little information is available on the active factors involved, and in many cases, accurate characterization of the mode of effect has not been possible.

Considerable work has been carried out with plants of the genus Lithospermum (Drasher and Zahl, 1946; Noble, Plunkett and Graham, 1951; Weisner and Yudkin, 1952). The inhibitory effects on hormone action of various extracts of L. ruderales have been difficult to measure. Breneman et al. (1960) developed an in vitro test and used cockerels as assay animals. The activity of LH, FSH and oxytocin was

found to be inhibited by extractives soluble in aqueous media.

About 1931, subterranean clover, var. Dwalganup, was introduced into Australia in an effort to develop and improve pasture land. Between 1941 and 1945, serious breeding problems were encountered with sheep grazing these pastures (Bennetts, Underwood and Shier, 1946). This syndrome is commonly referred to as "clover disease" (Moule, 1961), and the characteristic features are:

1. Poor conception in ewes despite repeated services.
2. Uterine inertia causing maternal dystocia.
3. Post-natal mortality with lambs.
4. Uterine prolapse and lactation in non-pregnant ewes.
5. Cystic glandular hyperplasia of the uterine endometrium.

Following the suggestion that problem pastures contained an estrogenic substance, bioassay of a crude ether extract administered orally to guinea pigs and mice elicited responses similar to those produced by injection of estradiol (Curnow, Robinson and Underwood, 1948). Subsequently, Bradbury and White (1951) isolated the estrogenic isoflavones formononetin and genistein from subterranean clover. Curnow (1954) recovered up to 740 mg genistein per 100 g D.M. Based on chemical analysis, this figure (7400 ppm) was thought to underestimate the actual content of some samples.

It had been demonstrated previously that the ability to stimulate estrus is by no means confined to substances

isolated from female reproductive glands. The occurrence in plants of estrogen-like substances was reported as early as 1926. A review of estrogens and related substances in plants was given by Bradbury and White (1954). Some constituents are identical with normal animal estrogens, while others possess structures widely divergent from the phenanthrene nucleus. Jensen (1962) commented on the structural requirements necessary for estrogenic activity and noted that they are quite general as compared to those of most biochemical processes. The molecular characteristics of all types of estrogenic compounds seem to be coplanarity and availability of pi electrons.

The importance of pasture plant estrogens in the reproduction and lactation of grazing animals was discussed by Pope (1954). In England, grasses were found to be active in early May. However, in most years no activity was found, and it was estimated that a cow grazing estrogenic pasture might ingest the equivalent of 1 mg estradiol per day for about six weeks. Estrogenic effects were reported in a flock of lambs grazing a red-clover pasture. Pope (1954) also stated that cases of reproductive or lactational abnormalities in grazing animals in Great Britain which might be attributed to plant estrogens are uncommon. These substances have been considered to be involved possibly in unexplained herd infertility in cattle, but little evidence is available to support this contention. Pope, McNaughton and Jones (1959) were able to show that most of the legumes and about

half of the grasses commonly found in British pastures have detectable quantities of estrogen as measured by mouse uterine weight bioassay.

In New Zealand, sheep grazing red clover-dominant pastures have shown effects of estrogenic stimulation. Coop and Clark (1960) were prompted to investigate a number of isolated reports that ewes grazed on alfalfa and red clover had poor lambing records. Investigation of ewes fed alfalfa showed that mating behavior was prolonged with a consequent later lambing period. It was postulated that the effects may have been due to estrogenic activity, or possibly to selenium toxicity. Ch'ang (1961) studied the reproductive performance of 253 female Romney sheep of different ages from 1958 to 1960. Comparisons were made between ewes grazed on red clover pastures and rye grass-white clover pastures. Ewe lambs on red clover accepted males before the normal breeding season, and such matings were infertile. These ewes at two years of age were unaffected. Six-year-old ewes had a protracted lambing season and a reduced lambing performance on red clover pasture. It was noted that the red clover was estrogenic during all periods, but no data were given to substantiate this statement. Munford and Flux (1961) estimated the relative activity of New Zealand red clover to be equivalent to 6000 to 7000 ppm genistein on a dry matter basis.

Schoop and Klette (1952) found estrogenic activity in German pasture plants and attributed herd sterility and

abnormal udder growth in cattle to the estrogenic content of these pastures. For the sexually-active material thought to be responsible for these reproductive disorders, Schoop (1952) proposed the name phytoestrogen. Rosenberger (1943) reported sexual disorders together with "Kohlenamie" in cabbage-fed cattle. Chury (1960) determined by mouse bioassay the estrogenic content of cabbage to be equivalent to 0.024  $\mu$ g estradiol benzoate per gram. It was suggested that the presence of activity in this plant could account for the sexual disturbances previously reported by Rosenberger (1943).

In the United States, Ladino clover has been under suspicion in this connection. Engle, Bell and Davis (1957) showed that ewes pastured on Ladino clover and birdsfoot trefoil conceived 22 and 20 days later, respectively, than those on bluegrass. Sanger, Engle and Bell (1958) confirmed by vaginal smears that estrogenic activity of Ladino and trefoil was involved. Recently (Sanger and Bell, 1961), Ladino clover feeding was shown to result in reduced fertilization of ova in ewes. In western Oregon a considerable area of red clover pastures are available to sheep during the breeding period. Fox and Oldfield (1958) observed that white-face, cross-bred ewes grazed on these pastures had a longer lambing season and lower lambing percentage than ewes fed red clover hay or alfalfa hay.

Since the initial isolation of genistein and formononetin from subterranean clover, various estrogenic

compounds have been found in a number of legumes. Three isoflavones have been isolated from red clover: biochanin A (Pope et al., 1953), formononetin (Bate-Smith, Swain and Pope, 1953), and genistein (Pope and Wright, 1954). Genistein, an isoflavone glucoside, was identified in soybeans by Walter (1941), and shown to be estrogenic by Cheng et al. (1953b). The aglucone part of this compound is genistein, and relatively large amounts, 0.1% as the glucoside, are present in soybean oil meal. A lactone, coumestrol, was isolated from Ladino clover by Bickoff et al. (1958). Recently, Guggolz, Livingston and Bickoff (1961) used chemical procedures to detect estrogenic isoflavones (daidzein, formononetin, genistein, biochanin A) and coumestrol in several forages. Their results are shown in Table 1.

TABLE 1  
Approximate Amounts of Estrogenic Compounds  
in Forages

(ppm in dry forage)

	Alfalfa	Ladino clover	Red clover	Subterranean clover
Daidzein	1	3	29	18
Formononetin	14	38	1700	900
Genistein	1	1	40	850
Biochanin A	1-5	0	1000	500
Coumestrol	57	49	18	26

Grundy (1957) stated that a fundamental aim of estrogen chemistry is to correlate biological activity and chemical composition. Such a task is no simple matter. Estrogenic potency of a compound depends on other factors besides superficial structure. Dosage, route and mode of administration, rate of absorption and destruction, in vivo biochemical modification, and sensitivity of the test animal are all important in determining the observed potency of a substance. These factors make various data on estrogen levels in forages [Bickoff et al. (1961), Cheng et al. (1953a), Chury (1960), Davies and Bennett (1962), Kitts et al. (1959), Munford and Flux (1961), Pieterse and Andrews (1956), Pope et al. (1959), and Robinson (1949)] very difficult to interpret.

Although forage estrogens are thought to be responsible for cases of reproductive disorders in sheep and perhaps in cattle, actual confirmation that these substances are the causal agents has not been possible. Isolation and identification of these compounds has led to an appreciation of their biological importance. However, quantities of pure compounds sufficient to carry out studies with large animals are not available at the present time.

It is well known that administration of exogenous estrogen to an animal disrupts the anterior pituitary mechanism and diminishes its capacity to secrete FSH (Turner, 1960). Underwood, Shier and Peterson (1953) found that diethylstilbestrol (DES), administered at 0.11 mg per day for six months to ewes, caused results similar to those



observed with subterranean clover feeding. The estrous cycle appeared normal, but almost complete infertility and dystocia were noted. Later, (Underwood et al., 1959), it was found that DES was effective in this respect until the dosage fell below 0.01 mg per day. Besides infertility and dystocia, persistent cystic changes in the endometrium resulted. However, Moule (1961) felt clover disease still had not been adequately investigated and that a quantitative approach to the production of plant estrogens, their intake and physiological response was necessary.

The effect of genistin on reproduction of Swiss mice was studied by Carter, Matrone and Smart (1955). Two diets containing 20 ppm and 10 ppm genistin respectively were fed to female mice from weaning through the time of reproduction. Premature vaginal opening indicated that the diets were estrogenic. The average food intake and weight gains on genistin and control diets were not significantly different. The reproductive data indicated that genistin lowered the number of animals littering, rather than the number of young per litter. However, this difference was not marked enough to be considered significant.

East (1955d) determined the effects of genistein on the fertility of the Fawn Farm strain of mice. Fifteen milligram genistein per mouse per day was administered in the diet.<sup>1</sup>

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<sup>1</sup> Assuming that an adult mouse consumes 5 g of diet per day, the concentration of genistein in the ration used by East (1955d) was about 3000 ppm.

Females were prefed for ten days on the diet and then allowed matings with untreated males. Eight of ten females mated immediately. Four matings were infertile, terminating in pseudopregnancy or fetal resorption. All second matings (presumably eight) were fertile, but a large number of stillborn embryos were noted at parturition. Males were prefed on the genistein diet for ten days also, and then mated with untreated estrous females. Five of ten males mated, and only two of these matings were fertile. These results led to the conclusion that males were more sensitive, but the exact assessment of the relative severity of the effects was complicated by the fact that females apparently became tolerant to genistein.

At present, no data have been published on the effects of coumestrol, biochanin A, formononetin, and daidzein on the fertility of laboratory animals. Booth (1959) stated that reproduction in mice was inhibited by coumestrol feeding, but that this was not true for rats. In a personal communication from Dr. Booth (1961), it was established that a diet containing 1250 ppm coumestrol acetate caused poor performance in mice. Diets were administered ten days prior to mating and during theoretical gestation. No litters were produced when treated males were mated with untreated females. Females receiving the coumestrol diet did cast litters when mated with untreated males.

Fox et al. (1957) reported on reproduction of mice fed diets containing 40% of either fresh red clover or red clover hay. The strain of mice employed was not indicated.

Males and females were prefed for 13 days, and then were allowed cohabitation according to a treatment plan for a subsequent period of 14 days. No litters resulted with females treated with red clover diets, whereas 27 of 30 controls produced litters. No conclusions could be made on the reproductive ability of males so treated. Red clover-treated females were able to maintain their body weight, and controls gained. Body weights of treated and control females at the time of mating were significantly different. Treated males lost about 2 g during the prefeeding period, whereas control males gained about 1 g. Recovery of fertility was observed when females were transferred to control ration. Hay-fed females seemed to recover faster than those previously fed the fresh clover diet. Further work by Fox et al. (1958) showed that red clover feeding in mice was difficult to interpret in terms of estrogenic or progestogenic effects or in terms of pituitary stimulation or inhibition.

Swierstra (1958) used U.B.C. Swiss mice to assess effects on reproduction brought about by feeding alcoholic extracts of red clover and birdsfoot trefoil. All of the extracts were estrogenic as measured by mouse uterine weight bioassay. Female reproductive efficiency, as indicated by the number littering, was greatly reduced after feeding extracts. Feeding DES at 0.068 ppm did not duplicate all of the effects produced by extract diets. Consumption of extract diets was significantly lower than control ration and DES diet. Also, body weights of extract-treated animals

was considerably below body weights of controls. However, this author apparently attributed the reproductive abnormalities to estrogenic substances in the clover and trefoil.

Nilsson (1961a) has recently shown that biochanin A labelled with tritium can be converted to genistein in the rumen fluid of sheep. Demethylation of biochanin A, producing genistein, was also shown to occur in the rat liver (Nilsson, 1961b). This author (1962) has developed a gel-filtration technique for separating conjugates and free metabolites of biochanin A from urine and feces of rats, but the results of this work have not been completely reported as yet.

Other than the account by Elghamry and Chury (1961), very little is known concerning the role that plant estrogens might play in influencing pituitary function. These authors found that estrogenic extracts from Ladino clover increased the LTH content of pituitary glands of male rats. Apparently, it has not been determined whether plant estrogens can influence FSH and LH production or release.

### III. MATERIALS AND METHODS

#### 1. LEGUME SAMPLES

Ladino clover (Trifolium repens) was obtained from the Piper-Newell field in Northwood, N. H., in 1960 and 1961. This field is about 800 feet above sea level and has a direct northern exposure. Van der Voet (1959) described the soil as Paxton loam, which is characteristic of the New England upland section of this region. The field was plowed in the spring of 1957 and harrowed throughout the summer. Two tons of lime per acre was applied in the spring, and in August, 1957, 800 lb per acre of 5-10-10 fertilizer was put on. Ladino clover was seeded August 17, 1957. In the spring of 1958, fertilization with 0-15-30 at the rate of 888 lb per acre was carried out. After the first crop was harvested, 0-15-30 was again put on at 666 lb per acre. The fertilization data are incomplete for following seasons, but it is known that light treatment with 0-15-30 was applied during 1960-61. The clover stand did not return in 1962.

The sampling history for the Piper-Newell field is outlined in Table 2. The 1960 samples were taken from the field at random. In April, 1961, nine plots were staked out at the western side of the field. The total area of the plots encompassed slightly over one-half acre. Random sampling of the plots was followed for all first growth material. The second growth clover was taken immediately adjacent to the plots.

TABLE 2  
Ladino Clover Samples - Piper-Newell Field

Sample No.	Date harvested	Description <sup>a</sup>	Height <sup>b</sup> (inches)
1	6/26/60	1G Bloom	9
2	6/26/60	2G Vegetative	3
3	8/--/60	3G Prebloom	-
4	5/1/61	1G Vegetative	1
5	5/15/61	1G Vegetative	4
6	5/30/61	1G Prebloom	7
7	6/12/61	1G Prebloom	10
8	6/19/61	1G Prebloom	10
9	6/26/61	1G Bloom	11
10	7/10/61	1G Bloom	12
11	7/24/61	1G Bloom	12
12	8/2/61	2G Prebloom	3
13	8/9/61	2G Bloom	5
14	8/16/61	2G Bloom	7

<sup>a</sup> 1G = first growth; 2G = second growth; 3G = third growth.

<sup>b</sup> Height of stand estimated in field at time of harvest.

The Pine Tree field is located in Durham, N. H., at an elevation of about 80 feet above sea level. The soil type is Buxton silt-loam. In 1959-60, corn was raised in preparation for seeding to legumes. The soil pH was 5.5, and 1.5 tons of lime was subsequently applied. In the spring

of 1961, alsike (Trifolium hybridum), red (T. Pratense), and Ladino clovers were seeded with oats as the companion crop. Twenty tons per acre of manure and 300 lb per acre of 8-16-16 fertilizer were administered at seeding. In the fall, an application of 0-15-30 was made at the rate of 300 lb per acre. After the first cutting in June, 1962, 300 lb per acre of 0-15-30 was applied to the field. The same treatment was given again in the fall of 1962. The early 1962 growth was predominantly Ladino, but particular care was taken during collection of samples to exclude red and alsike clover. The sampling history is outlined in Table 3.

TABLE 3

## Ladino Clover Samples - Pine Tree Field

Sample No.	Date harvested	Description <sup>a</sup>	Height <sup>b</sup> (inches)
15	5/4/62	1G Vegetative	3
16	5/5/62	1G Vegetative	3
17	5/10/62	1G Vegetative	5
18	5/19/62	1G Prebloom	7
19	5/26/62	1G Prebloom	9
20	5/30/62	1G Prebloom	12
21	6/22/62	2G Bloom	4
22	6/25/62	2G Bloom	6
23	7/2/62	2G Bloom	7

<sup>a</sup> 1G = first growth; 2G = second growth.

<sup>b</sup> Height of stand estimated in field at time of harvest.

Alfalfa (Medicago sativa) and Ladino clover were obtained in 1962 from the Price field in Northwood, N. H., for estrogen determinations (Table 4). This was a cooperative venture with the Agronomy Department of the University of New Hampshire. A description of this field is given by Peterson et al. (1961).

TABLE 4  
Other Forage Samples

Sample No.	Forage	Date harvested	Description <sup>a</sup>	Height <sup>b</sup> (inches)	Source <sup>c</sup>
24	Alfalfa hay	7/--/61	Barn dried and baled	-	JHF
25	Alfalfa	9/21/61	4G Vegetative	6	JHF
26	Alfalfa	6/15/62	2G Vegetative	4	JHF
27	Alfalfa	6/19/62	1G Bloom	15	PF
28	Alfalfa	7/10/62	2G Vegetative	7	PF
29	Ladino	6/29/62	1G Bloom	12	PF
30	Ladino	6/19/62	1G Bloom	10	PF
31	Timothy grass	9/14/62	Pasture-Vegetative	8	UNH

<sup>a</sup> 1G = first growth; 2G = second growth; 4G = fourth growth.

<sup>b</sup> Height of stand estimated in field at time of harvest.

<sup>c</sup> JHF = Juniper Hill Farm; PF = Price field; UNH = University of New Hampshire Dairy Department.



The herdsman, Mr. Wendell Wells, at the Juniper Hill farm in Canterbury, N. H., supplied samples of alfalfa suspected of contributing to breeding problems in a Holstein herd. The field where the forage was grown is located in the Merrimac River valley at an elevation of 280 feet. The soil here was of the Ondawa series of the fine sandy-loam variety as described by Van der Voet (1959). The details of fertilization are uncertain, but large amounts of commercial mineral fertilizer were applied in 1961. A sample of third crop barn-dried alfalfa hay was analyzed for estrogenic activity, and some of this material was tested in Extract Reproduction Experiment II. Additionally, bioassays were carried out on fourth growth vegetative alfalfa taken from this locale in September, 1961. In June, 1962, second-growth, vegetative material was taken for estrogen bioassay. Little fertilizer was applied during the second growing season, and the soil was especially dry at the time of sampling. A description of the aforementioned samples is given in Table 4.

Samples were harvested only on fair days. Rough estimates of growth and stage of maturity were made in the field at each harvest, and previous adverse weather conditions were also noted. The aerial portion of the plant was severed as close to the ground as possible and was removed by hand to prevent inclusion of roots, grass and other foreign material in the sample. The samples were transported in sealed paper bags to the laboratory and immediately treated.

The method of treatment, of course, varied with the experimental design.

For dry samples, the plants were spread loosely in 3x10x24-inch, cloth-lined wire baskets and placed in a Blue M, model no. FC572, mechanical convection oven at 60 C. The length of drying time varied with different samples, but was usually 6 to 12 hours. The dried samples were immediately pulverized in a Wiley Mill to a maximum particle size of one millimeter. Storage of plant powder was somewhat variable. 1960 Ladino clover (sample 3) was sealed at room temperature in a round cardboard container. The 1961 alfalfa and Ladino samples were held in sealed one-gallon glass jars prior to experimental use. The best preservation was obtained with the method used during 1962, which involved placing samples in metal two-pound coffee cans, sealing with masking tape, and storing in a freezer at -18 C.

Fresh samples were extracted as quickly as possible. When large amounts were used, excess material was retained at 4 C in a refrigerator until extraction could be accomplished.

Measurements of leaflet and petiole composition of 1962 Ladino samples are given in Table 5. Two 100 g portions of healthy, fresh leaf were randomly selected in the laboratory. One portion, intact leaf, was air-dried in the convection oven for six hours. The leaflets and petioles were separated by hand in the other 100 g portion. The

fresh weights of these were taken, the material then dried concurrently with the leaf portion, and the dry weights of leaflet, petiole, and intact leaf determined after a one-hour cooling period under normal atmospheric conditions.

Proximate analyses of several forage samples were done by the Biochemistry Department of the University of New Hampshire. Table 6 lists the results of analyses on Ladino from the Piper-Newell field, alfalfa from the Juniper Hill farm, and timothy grass (Phleum pratense).

TABLE 5

Relative Amounts of Leaflet and Petiole in First Growth  
Clover Samples: Pine Tree Field

Date	Air dry wt leaf (100 g fresh wt)	Fresh leaf		Air dry leaf	
		Leaflet %	Petiole %	Leaflet %	Petiole %
5/4/62	14.2 g	58	42	70	30
5/10/62	15.5 g	58	42	69	31
5/18/62	14.3 g	50	50	67	33
5/25/62	10.3 g	35	65	53	47
5/31/62	13.2 g	30	70	44	56
6/9/62	10.3 g	27	73	44	56

TABLE 6  
Proximate Analysis of Forages<sup>a</sup>

Forage	Sample No.	Ash	Fat	Fiber	Protein	N.F.E. <sup>b</sup>
Ladino	3	10.60	2.70	17.09	25.60	44.01
Ladino	5	9.92	3.83	10.01	30.12	46.12
Ladino	6	10.02	3.60	13.75	28.77	43.86
Ladino	8	8.82	3.15	14.75	26.08	47.20
Ladino	10	8.51	2.87	16.76	22.20	49.66
Ladino	11	9.26	2.90	18.25	24.03	45.56
Ladino	12	7.97	3.25	13.51	26.19	49.08
Ladino	14	8.29	2.85	17.96	22.29	48.61
Alfalfa hay	24	9.58	2.92	27.32	22.85	37.33
Alfalfa	25	11.29	3.05	13.68	33.94	38.04
Timothy	31	8.69	5.17	28.51	18.08	39.55

<sup>a</sup> Results are in percentage dry matter.

<sup>b</sup> N.F.E. = nitrogen free extract.

## 2. ANIMALS AND MANAGEMENT

All experiments in 1960 were done with SWR/Jax mice obtained from the production department of the Jackson Memorial Laboratory, Bar Harbor, Maine. Since this is an inbred strain, body weights and size of litters were less than would be expected in other hybridized laboratory stocks. Reproduction Trial I, Vaginal Smear Experiment I, and bioassay studies with 1960 Ladino samples were carried out with animals produced from a nucleus of breeders purchased July 21, 1960.

The majority of the experiments performed after 1960 involved the use of CD-1 mice from the Charles River Breeding Laboratories, Brookline, Massachusetts. The original strain was the HaM/ICR (Hauschka and Mirand-Institute). The CD-1 strain was derived out of this initial stock from gnotobiotic animals delivered by caesarean section in sterile plastic isolators. "Caesarean-originated, barrier-sustained" animals were shipped upon request, and no attempt was made to maintain a colony.

The proposals for raising laboratory mice and rats by Eaton and Cabell (1949) provided the foundation of the animal care program. A well-ventilated room situated on the ground floor of the Ritzman Laboratory at the University was used as the animal room. The room has a concrete floor, containing a drainage duct, and facilities for holding cages

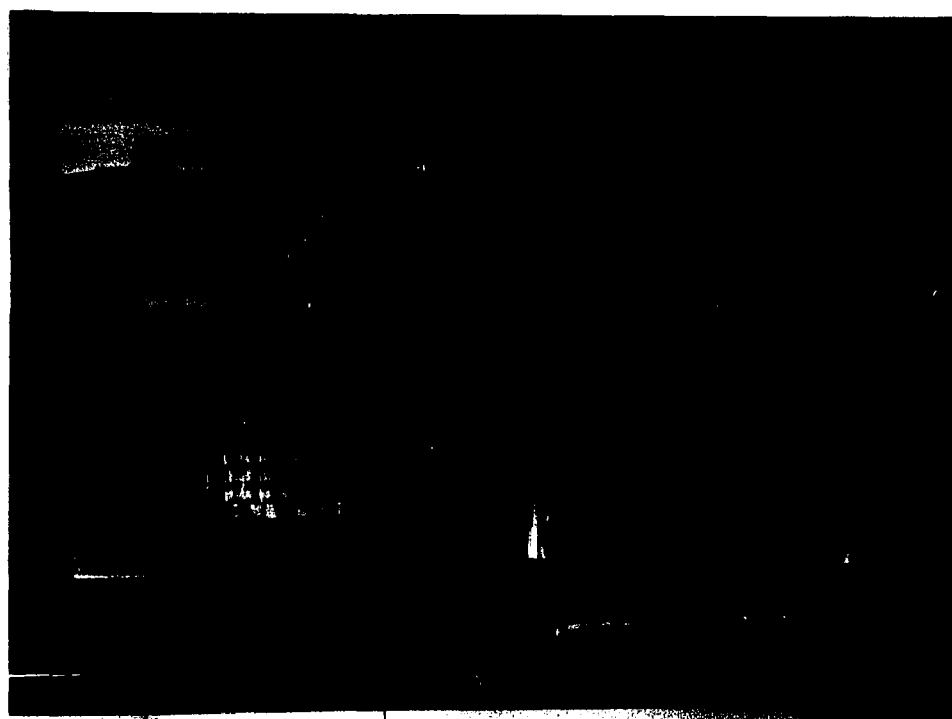
well above the floor. The shelves and floor were periodically cleaned and disinfected as standard laboratory procedure. No artificial lighting device was used, but care was taken in all reproduction experiments to maintain normal amounts of illumination. Forced ventilation was continuous throughout, and, as a result, a relatively stable air temperature was obtained (average = 21 C).

Cages constructed with plywood sides and metal bottoms were used. Outside dimensions of these were 7.75 inches wide, 10.75 inches long, and 5.5 inches deep. The tops were 1/4-inch mesh hardware cloth enclosed in a wooden frame of dimensions permitting a snug fit on cages. Food hoppers were present in all tops for holding pelleted food. These were made from 3/8-inch mesh hardware cloth and allowed easy access to food. For some of the experiments, wooden platforms were installed in a corner of the cage so that feeding dishes placed inside the cages were held above the cage bottom. This arrangement greatly reduced the likelihood of contamination of the experimental rations by the animals.

Special modifications were made in cages used for digestion studies with crude forage diets. Figure 1 shows this apparatus. The metal bottoms of regular cages were replaced with 1/2-inch mesh hardware cloth. Below this, an inclined sheet of nylon screening was incorporated to catch feces and divert them to a metal collection pan. Urine passed through the screen and was absorbed on paper towels

placed beneath the cages. Feeding platforms, previously described, were two inches above the wire cage bottom. A piece of 1/2-inch mesh hardware cloth was placed on top of the feeding dish. This acted as a barrier to the animals, and food could not easily be dislodged over the rim of the dish. The results of the digestion study are shown in the Appendix.

Fig. 1. Digestion Cages



Top view

End view

See text for description.

Water was provided ad libidum in inverted bottles with a glass tube extending into the cage. The bottles had a capacity of 120 ml, and the drinking tubes were 1/4-inch

in diameter. The bottles were cleaned at two-day intervals and fresh water was replaced whenever contamination was observed. Clean, dry shavings were used to cover cage bottoms. Usually, a maximum of four adult animals was allowed per cage. Under these conditions, it was found advisable to clean cages every five days. All cages were periodically scrubbed with hot disinfectant solution and allowed to dry in direct sunlight.

Body weights were determined on a triple beam balance with a weight capacity of 610 g. Weights were always taken to the nearest tenth of a gram. A one-liter beaker was used to contain the animal during weighing. The recorded figure was taken while the animal was motionless in the weighing container.

Whenever pregnancies were observed, the animal was isolated at about day 16 of gestation. A tuft of cotton batting was placed in the cage from which a well-insulated nest could be fabricated. Not more than ten young per litter were allowed per lactating female. In some litters of the CD-1 strain, numbers larger than this resulted. In these instances, runts were sacrificed as early as possible. The young were weaned at least by 18 days, and then segregated according to sex.



### 3. EXTRACTION TECHNIQUES AND APPARATUS

Since there are a multitude of methods for extracting forage estrogens and other substances with biological activity, certain aspects of different ones were selected for specific purposes. Reference will be made to methods already in the literature, and variations, when used, will be described in more detail.

In forage estrogen determinations, the method of Bickoff et al. (1959) was followed for extracting fresh and dried samples. Figure 2 depicts equipment used to prepare fresh samples, and figure 3 shows the Soxhlet extractors employed for dry material.

The methods used in isolating fractions for Interaction Experiment I are outlined in figure 4. The acetone procedure is similar to that used for preparing extracts for bioassay. The method for saponin separation was modified slightly from the one given by Wall et al. (1952). The fresh plant was macerated in a Waring blender in the first step instead of being ground in a Ball and Jewell mill. It had been recommended that benzene extraction be carried out in a continuous liquid-liquid extractor, but good results were obtained by simply extracting in a separatory funnel.

The scheme for Extract Reproduction Experiment I can be seen in figure 5. Here, too, acetone extraction was similar to that of the bioassay procedure. The idea for

Fig. 2. Equipment for Fresh Forage Extraction



Fresh plant was chopped with the paper cutter and then macerated with solvent in the blender for 30 seconds. The homogenate was filtered in the large Büchner funnel. The filtrate was stored in a sealed flask until concentration and diet preparation could be accomplished.

Fig. 3. Soxhlet Apparatus for Extracting  
Dried Samples



A sand bath provided an operating temperature of 78 C. About 60 g of dried pulverized legume could be added to the extraction chamber. A small wisp of glass wool was used to cover the flushing tube exit in the extracting chamber.

Fig. 4. Extraction Scheme for Interaction Experiment I

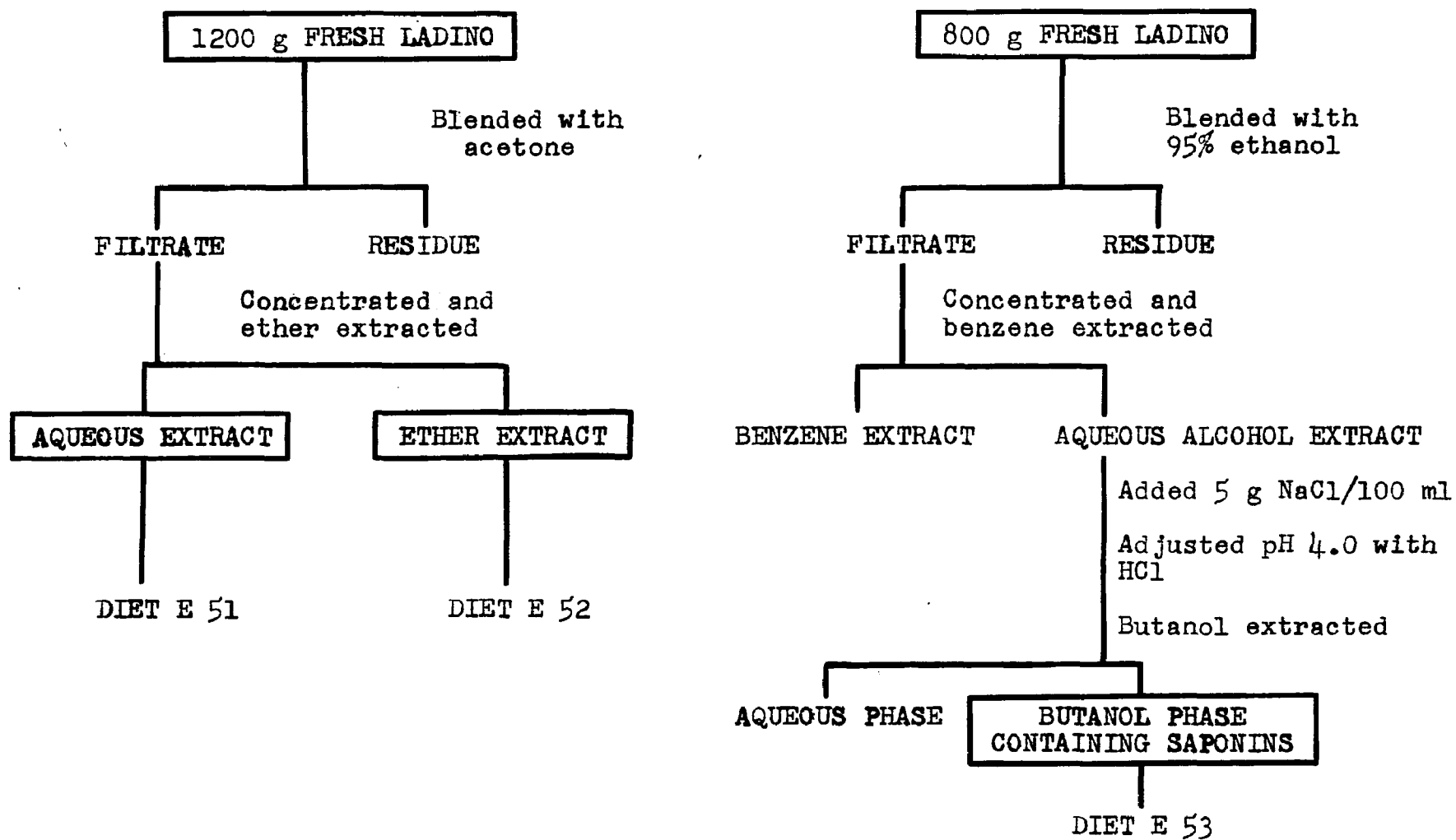
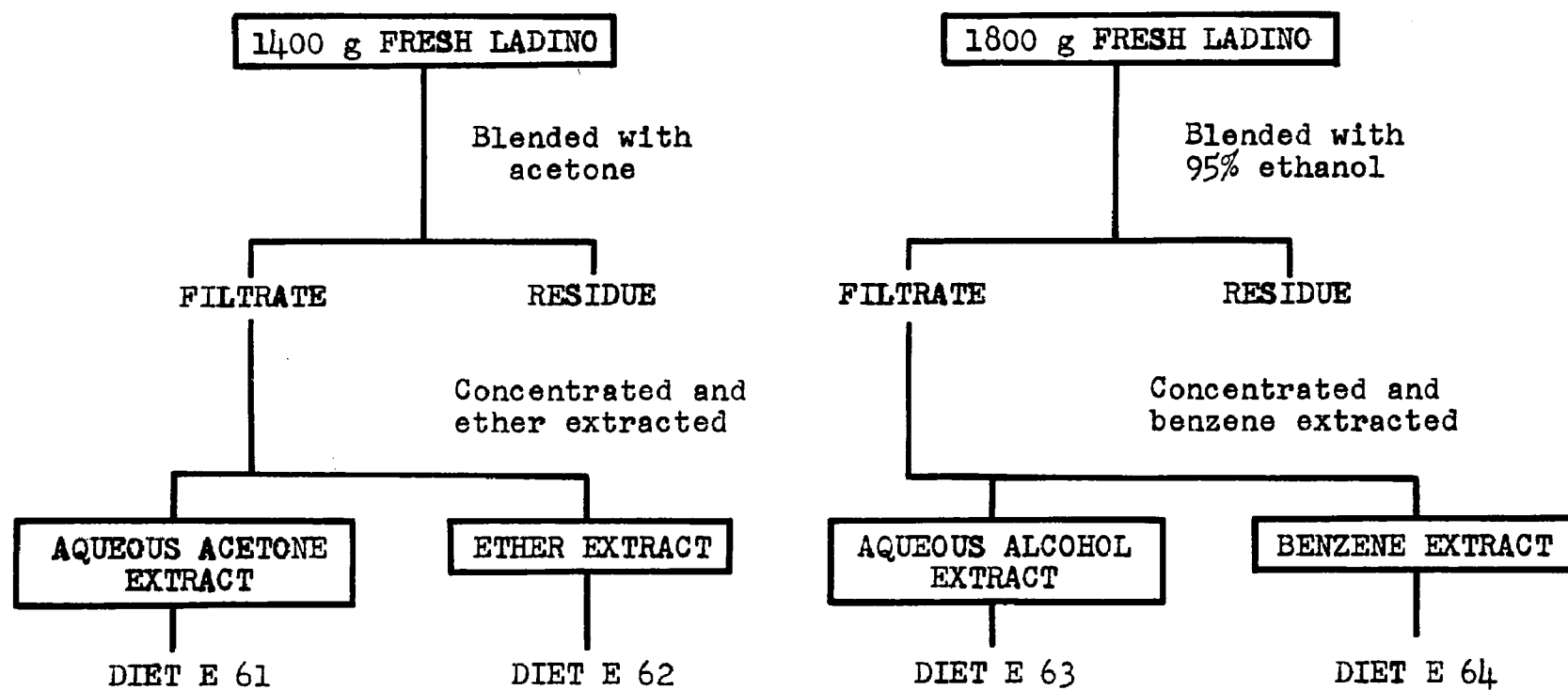


Fig. 5. Extraction Scheme for Extract Reproduction Experiment I



the ethanol method was taken from Robinson (1949). Alkali was not used because Pieterse et al. (1956) have shown that the estrogenic activity of genistein is increased by this treatment. It is suspected that other flavonoids react in a similar manner (Pope et al., 1959). This would undoubtedly lead to an excessively high "apparent estrogen" content. Benzene extraction of the aqueous ethanolic concentrate was decided upon because Curnow (1954) found that only 5% of the isoflavonoid estrogenic activity was removed in this way. Furthermore, Ostrovsky and Kitts (1962) have shown that benzene fractionation removes inhibitory material(s) which influence uterine response in estrogen bioassay.

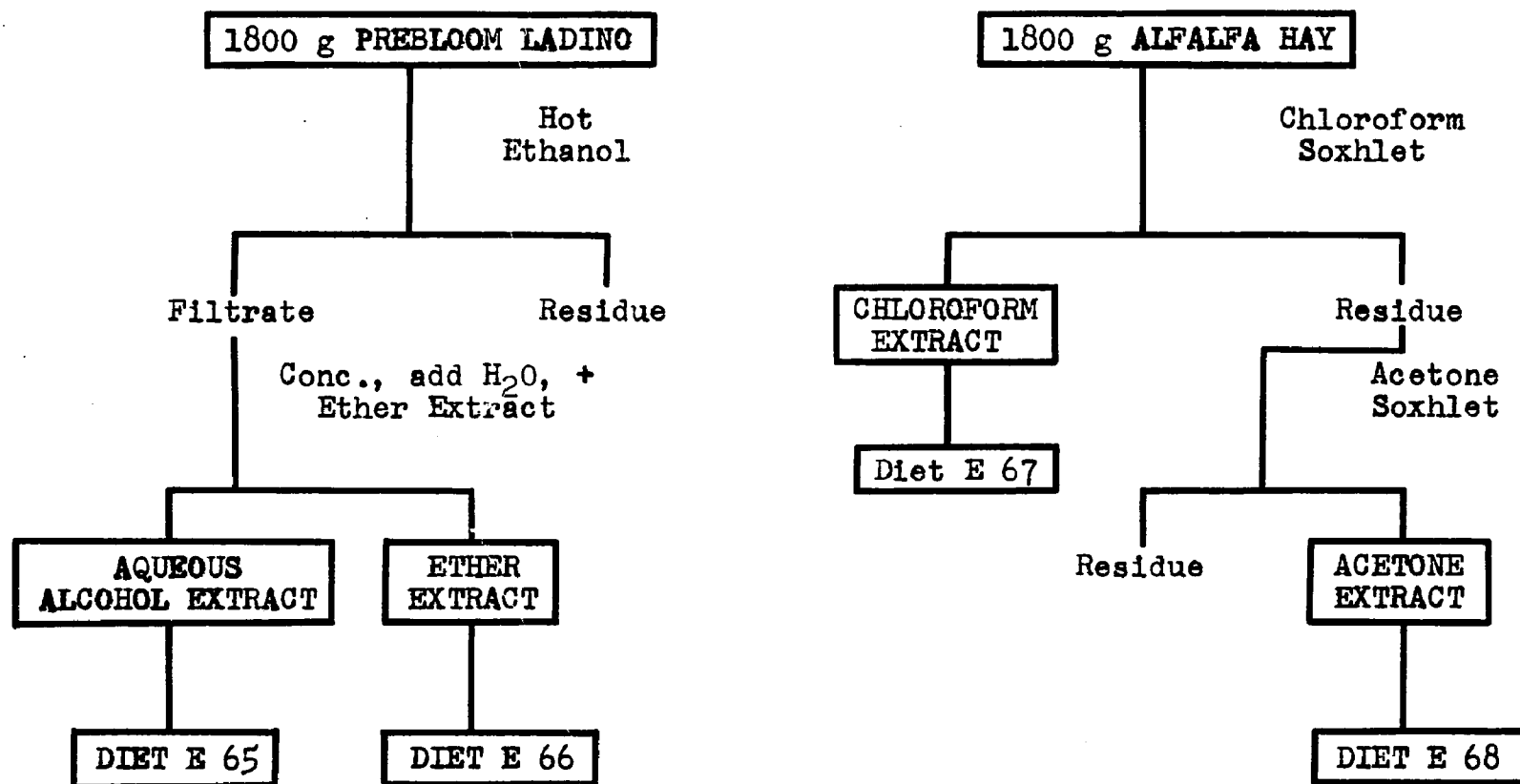
The extraction outline in figure 5 was designed to allow a comparison of the two main techniques employed for the removal of estrogenic activity from forages. The chief objective was to determine which fractions contain substances detrimental to female reproductive processes and thus to correlate such effects with estrogenic activity. The theory in this case was that the solvent properties of ether and benzene are similar, as are acetone and alcohol. Ether removes estrogen-like material, whereas benzene does not. Therefore, reproductive disorders should result from the administration of ether extract and aqueous-alcohol extract if estrogens are the causal agents. Any other results would be presumed to be a manifestation of other factors working alone or possibly in combination with estrogens.

Two different dried forages were compared in Extract Reproduction Experiment II, and the fractionation outline is shown in figure 6. A low-estrogen Ladino sample was first refluxed with ethanol as described by Kitts et al. (1959). Further purification was attempted by subsequent ether extraction of an aqueous alcoholic concentrate. Ether was selected as the solvent because Bickoff et al. (1959) found it to be more selective for estrogens, primarily coumestrol, in Ladino clover. Moreover, Curnow (1954) found that 95% of the isoflavonoid estrogens were removed by ether in a method very similar to this.

Alfalfa hay with high estrogenic activity was fractionated according to a suggestion made by Bickoff et al. (1959). Considerable material was first removed by extracting with chloroform in a side chamber Soxhlet apparatus. The dried residue was again extracted in the Soxhlet device with acetone. The acetone dissolved much less from the hay than did the chloroform. Figure 7 is a photograph of the side chamber Soxhlet apparatus used in the preparation of these alfalfa fractions. This apparatus was also employed with samples involved in Weanling Experiment IV and the Transplant Experiment.

Since the literature contains no standardized instructions on the use of the side chamber Soxhlet apparatus in this type of work, the method used in these studies will be explained in more detail. A cloth bag is sometimes suggested for enclosing the sample during extraction, but

Fig. 6. Extraction Scheme For Extract Reproduction Experiment II

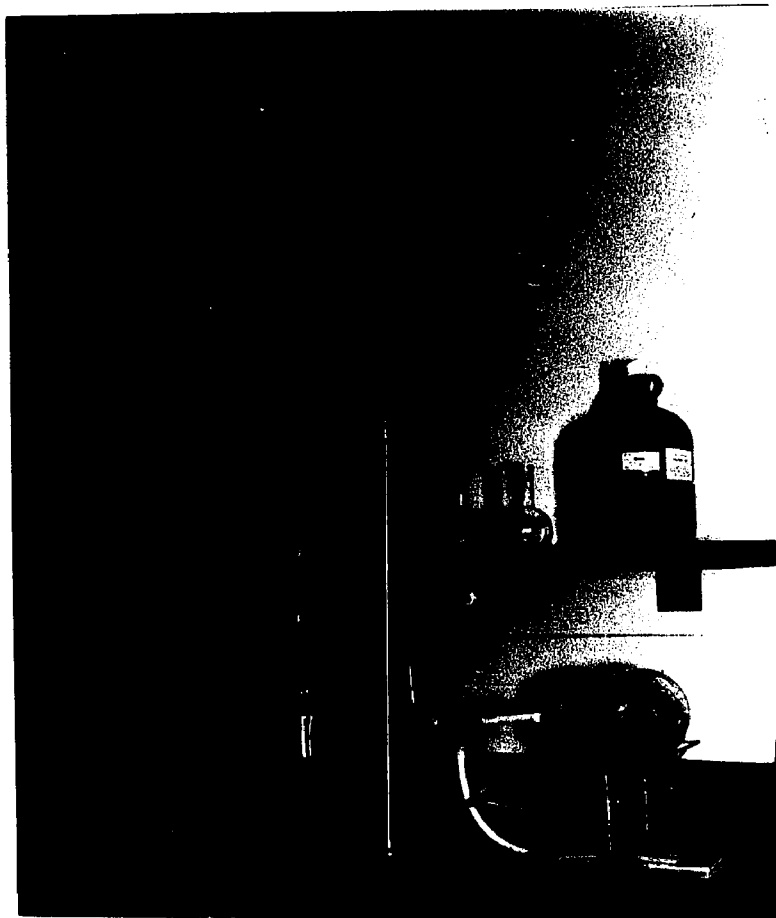




this was found to be unnecessary. Actually, a larger sample could be handled without it. The bottom of the side chamber was first packed with glass wool to retain the sample and to prevent contamination of the extract. Then the slotted glass plate was put in place, and the sample was added directly onto the plate. The side chamber was filled to the height of the top of the siphon tube. Usually 600 to 800 g of dried pulverized plant could be packed into the chamber if the sample was tamped gently with a glass rod while loading. Two liters of solvent were added to the flask and enough solvent added to the side chamber to completely cover the sample (about one liter). Temperature regulation of the water bath depended on the solvent involved. Running temperatures were selected such that the chamber would be refilled with solvent 20 minutes following siphoning. All extractions were run for a standard operating time of 24 hours.

It was intended that the results of the Extract Reproduction Experiments would allow a comparison of the relative potency of fresh and dried samples. Extract Reproduction Experiment II was an attempt to determine differences between high and low estrogen legumes. The extraction scheme was planned so that a rather pure estrogen fraction (the alfalfa acetone extract) could be tested separately. Results produced by this fraction could then be compared with the effects of other fractions. It was anticipated that substances of inhibitory nature, such as those described by Bickoff et al. (1960) and Ostrovsky and Kitts (1962) in connection with

Fig. 7. Side Chamber Soxhlet Apparatus



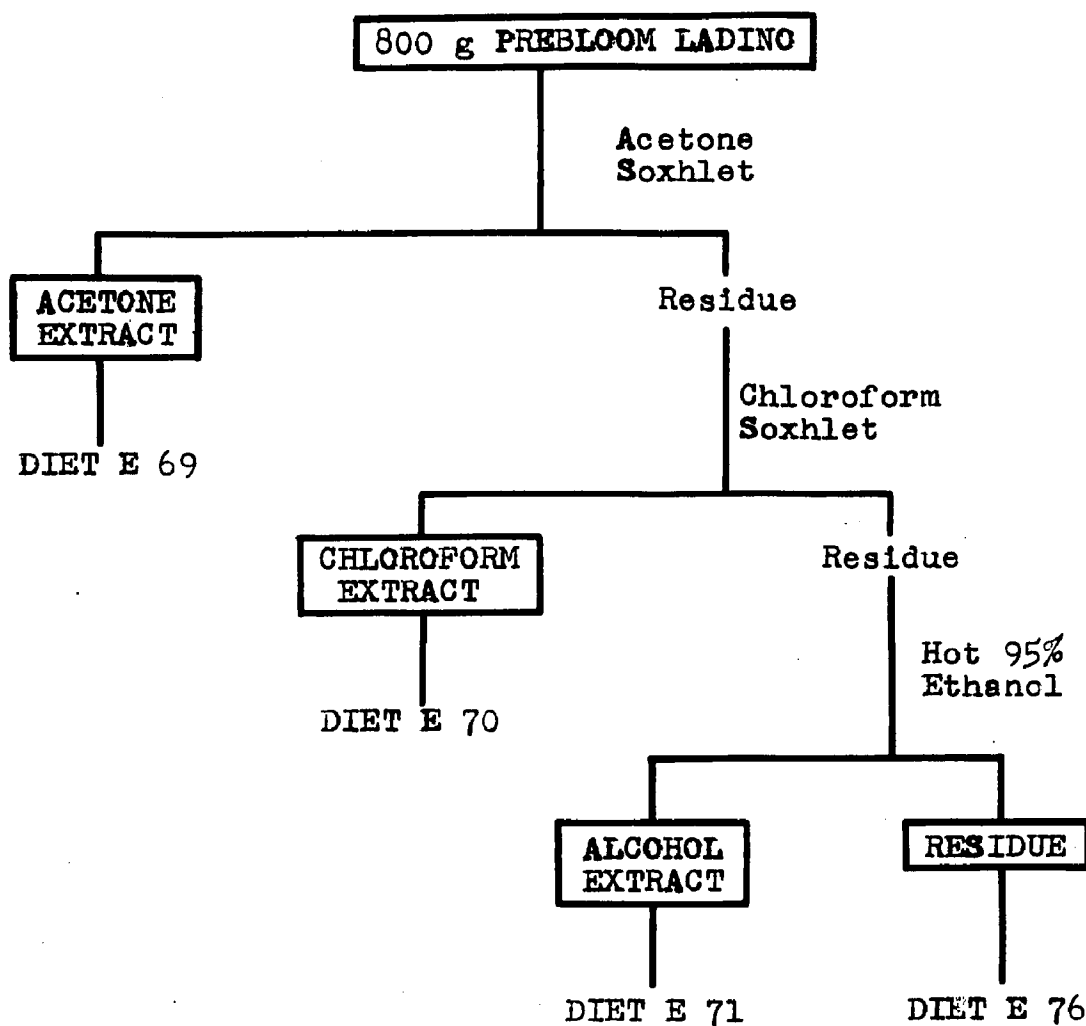
Water-leveling device is shown attached to the water bath. See text for procedure used.

uterine response in bioassay animals, would occur in the Ladino ether extract and the alfalfa chloroform extract. Therefore, depending on the results, a fairly accurate estimation of the nature of the materials involved would be possible.

Weanling Experiments I, II, and III were carried out with crude forage diets, and many of the results were difficult to interpret in terms of specific activity of certain compounds in the samples. Therefore, a Ladino prebloom sample possessing inhibitory characteristics as measured by Weanling Experiment III was selected for study in Weanling Experiment IV. The origin of the four fractions involved is explained in figure 8. Since a chloroform extraction prior to use of acetone removes considerable "inhibitory" activity (Bickoff et al., 1959), it was felt that a reversal of this sequence might result in a more useful purification of the biologically active substances. A final alcoholic extraction was incorporated to allow separation of additional material from the residue. Some doubt still remains as to whether acetone is especially useful for the removal of estrogens other than coumestrol. This idea also supports the inclusion of alcohol in this particular scheme.

Vegetative Ladino, sample no. 15, was studied in the Transplant Experiment. A 700 g sample was acetone extracted in the side chamber Soxhlet apparatus. Another 700 g portion of the same sample was extracted with chloroform. These two fractions were tested separately.

Fig. 8. Extraction Scheme For Weanling Experiment IV



#### 4. DIETS AND THEIR PREPARATION

The procedure for preparing diets for bioassay of estrogenic activity was taken from Bickoff et al. (1959). With fresh forage, the initial acetone extract was further purified by ether extraction. A single acetone extraction was made with dried samples. The final extracts were concentrated under reduced pressure, and the consolidated fluid was then added to basal ration. The basal ration in bioassay determinations was finely-ground Purina laboratory chow. The solvent in the extract-basal ration mixture was evaporated at room temperature under vacuo. Final traces of the solvent were removed under an air current for 24 hours. The equipment used in preparing these and all other diets compounded from organic solvent extracts is shown in figures 9 and 10.

In one part of the bioassay work, the water soluble phase (acetone-aqueous) resulting from ether extraction was tested for estrogenic activity. This solution was a somewhat viscous, amber-colored liquid. Concentration of it was carried out under reduced pressure at 60 C. The concentrated acetone-aqueous phase was then added to basal ration, and the resultant mash was dried in the convection oven at 60 C. The dry diet was then cooled to room temperature and immediately pulverized in a large cast-iron mortar and pestle. These particular diets are listed in Table 8,

Fig. 9. Extract Concentrating Apparatus



For larger quantities of extract, 3000 ml flasks were used. Extracts prepared as shown in figure 3 were concentrated in 500 ml flasks. A rotating evaporator was employed in all cases. A water-filled ballast jar was included between the evaporator and vacuum pump for safety purposes and to aid in the removal of water soluble solvents.

Fig. 10. Diet Preparation Apparatus



Extract diets were stored under refrigeration in sealed, amber glass bottles. During feeding periods, diets were contained in wide-mouth one-gallon jars. Legume samples were stored in 2-lb coffee cans. Commercial corn oil was used in the preparation of DES diets.

Large bell-covered dessicators were used in evaporating solvents from extract-diet mixtures. The bottom portion was employed when final drying was brought about under an air current.

A glass pestle is shown in the lower center portion of the photograph.

whereas all of the regular bioassay diets are given in Table 7.

Many different diets were tested in the various parts of the experimental procedure. In order to facilitate reference to specific ones, each diet was assigned a number. Since many of the rations were related as to method of preparation and/or composition, letters were delegated to further identify these in this respect. The control or basal ration in every experiment was powdered Purina laboratory chow. Throughout the text, this will be referred to as diet 1 and is listed in this way at the tops of Tables 7, 8, 9, and 11.

The routine for extracting fresh and dried forage samples is described in the preceding subsection. Experimental extract diets were prepared by mixing concentrated extracts with basal ration. Excess organic solvent was removed under vacuum in the apparatus shown in figure 10. An air current was drawn over the diet for 24 hours to remove final traces of organic extractants. With aqueous extracts, the concentrate-basal ration mixture (wet mash) was dried in the convection oven at 60 C. During the 24-hour final drying period, all diets were frequently mixed to insure a uniform distribution of additives in the ration. Usually, large amounts of experimental extract diets (1000 to 2000 g) were prepared. As a result, a flat-bottom pestle (figure 10) was found to be useful for stirring the mixture and crushing lumps. Many of the extracts had a tendency to form tarry deposits on the top of the diet mixture if this precaution were not taken.



TABLE 7

## Bioassay Diets

Diet no. <sup>a</sup>	Equivalence <sup>b</sup>	Source of extract <sup>c</sup>	Forage sample no. <sup>d</sup>
1 <sup>e</sup>	0	----	---
B2	1	Fresh 1G Ladino	1
B3	1	Fresh 2G Ladino	2
B4	0.1	Dried 2G Ladino	2
B5	0.06	Dried 2G Ladino	2
B6	2	Fresh 1G Ladino	4
B7	2	Fresh 1G Ladino	5
B8	2	Fresh 1G Ladino	6
B9	2	Fresh 1G Ladino	6
B10	2	Fresh 1G Ladino	7
B11	2	Fresh 1G Ladino	9
B12	2	Fresh 1G Ladino	10
B13	2	Dried Alfalfa Hay	24
B14	4	Fresh 4G Alfalfa	25
B15	1	Dried Alfalfa Hay	24
B16	1	Dried 4G Alfalfa	25
B17	1	Dried 1G Ladino	15
B18	1	Dried 1G Ladino	17
B19	1	Dried 1G Ladino	18
B20	1	Dried 1G Ladino	19

TABLE 7 (continued)

Diet no. <sup>a</sup>	Equivalence <sup>b</sup>	Source of extract <sup>c</sup>	Forage sample no. <sup>d</sup>
B21	0.6	Dried 1G Ladino Leaflet	16
B22	0.6	Dried 1G Ladino Leaflet	17
B23	0.6	Dried 1G Ladino Leaflet	18
B24	0.6	Dried 1G Ladino Leaflet	19
B25	1	Dried 2G Alfalfa	26
B26	1	Dried 1G Alfalfa	27
B27	1	Dried 2G Alfalfa	28
B28	1	Dried 1G Ladino	29
B29	1	Dried 1G Ladino	30
B30	1	Dried 2G Ladino	23
B31	1	Dried 2G Ladino	23
B32	1	Dried 2G Ladino	23
B33	2	Fresh 2G Ladino	23

<sup>a</sup> B indicates bioassay diet.

<sup>b</sup> See text for an explanation of equivalence.

<sup>c</sup> 1G = first growth; 2G = second growth; 4G = fourth growth.

<sup>d</sup> Further description of these samples is given in Tables 2, 3, and 4.

<sup>e</sup> Diet 1 = Control or basal ration.

TABLE 8

## Experimental Extract Diets

Diet no. <sup>a</sup>	Equivalence <sup>b</sup>	Extractant	Forage Extracted <sup>c</sup>	Forage sample no. <sup>d</sup>	Experiments involved
1 <sup>e</sup>	0	---	0	--	all
E51	2	Acetone-Aqueous	Fresh 1G Ladino	10	Interaction Experiment I
E52	2	Ether	Fresh 1G Ladino	10	Interaction Experiment I
E53	2	Saponin	Fresh 1G Ladino	10	Interaction Experiment I
E54	2	Acetone-Aqueous	Fresh 1G Ladino	4	Bioassay <sup>f</sup>
E55	2	Acetone-Aqueous	Fresh 1G Ladino	5	Bioassay <sup>f</sup>
E56	2	Acetone-Aqueous	Fresh 1G Ladino	6	Bioassay <sup>f</sup>
E57	2	Acetone-Aqueous	Fresh 1G Ladino	6	Bioassay <sup>f</sup>
E58	2	Acetone-Aqueous	Fresh 1G Ladino	7	Bioassay <sup>f</sup>

TABLE 8 (continued)

Diet no. <sup>a</sup>	Equivalence <sup>b</sup>	Extractant	Forage Extracted <sup>c</sup>	Forage sample no. <sup>d</sup>	Experiments involved
E59	2	Acetone-Aqueous	Fresh 1G Ladino	9	Bioassay <sup>f</sup>
E60	2	Acetone-Aqueous	Fresh 1G Ladino	10	Bioassay <sup>f</sup>
E61	1	Aqueous-Acetone	Fresh 1G Ladino	11	Extract Reproduction Experiment I
E62	1	Ether	Fresh 1G Ladino	11	Extract Reproduction Experiment I
E63	1	Aqueous-Ethanol	Fresh 2G Ladino	14	Extract Reproduction Experiment I
E64	1	Benzene	Fresh 2G Ladino	14	Extract Reproduction Experiment I
E65	1	Aqueous-Ethanol	Dried 1G Ladino	5,6,7,8 (pooled)	Extract Reproduction Experiment II
E66	1	Ether	Dried 1G Ladino	5,6,7,8 (pooled)	Extract Reproduction Experiment II
E67	1	Chloroform	Dried Alfalfa Hay	24	Extract Reproduction Experiment II

TABLE 8 (continued)

Diet no. <sup>a</sup>	Equivalence <sup>b</sup>	Extractant	Forage Extracted <sup>c</sup>	Forage sample no. <sup>d</sup>	Experiments involved
E68	1	Acetone	Dried Alfalfa Hay	24	Extract Reproduction Experiment II
E69	0.8	Acetone	Dried 1G Ladino	20	Weanling Experiment IV
E70	0.8	Chloroform	Dried 1G Ladino	20	Weanling Experiment IV
E71	0.8	Ethanol	Dried 1G Ladino	20	Weanling Experiment IV
E72	2	Acetone	Dried 1G Ladino	15	Transplant Experiment
E73	2	Chloroform	Dried 1G Ladino	15	Transplant Experiment

<sup>a</sup> E indicates experimental extract.

<sup>b</sup> See text for an explanation of equivalence.

<sup>c</sup> 1G = first growth; 2G = second growth.

<sup>d</sup> Further description of these samples is given in Tables 2, 3, and 4.

<sup>e</sup> Diet 1 = Control ration.

<sup>f</sup> These diets contained the aqueous-acetone phase remaining after ether treatment in the bioassay extraction procedure.

Table 8 gives a complete list of experimental extract diets. Special note should be made concerning diets E61 and E63, for in these, aqueous extracts were not incorporated as previously described. Instead, the aqueous solutions were stored in stoppered flasks at 4 C, and aliquots of each were mixed with basal ration at each feeding. The volume of the aliquots was adjusted to provide the equivalence listed in Table 8.

The quantification of the measure of amounts of materials in extracts incorporated in the diet needs some clarification. This undoubtedly results from the fact that the substances removed in the extract are of unknown composition. Hence, the usual procedure of indicating concentrations per unit volume (or weight) cannot be used. Therefore, a method of equivalence was adopted. Extracts were added to basal ration so that the extractives from a known weight of original plant material would be contained in a certain weight of the resultant diet. The concentration of extractives in the diet was related on the basis of the ratio of the weight of material processed to the weight of the diet. Equivalence, as used here, equals the quotient of the grams of sample extracted divided by the grams of the ration in which the extract is incorporated.

$$\text{Equivalence} = \frac{\text{grams forage extracted}}{\text{grams diet}}$$

This term provides for an evaluation of the equality of certain effects, but does not allow a determination of

values in quantitative terms. For example, an extract diet prepared from fresh forage with equivalence of 1.0 contains much less plant material than does a diet similarly prepared from dried forage with the same equivalence. This discrepancy in actual amounts is not unwarranted, however, because results from either fresh or dried forages can be applied to actual situations. Possible effects on farm animals from either pasture legume or legume in hay, for instance, could more easily be deduced from the system of equivalence than from a knowledge of the actual amounts of extractives taken from the forage.

Crude forage diets were compounded by adding dried legume meal to powdered basal ration. Table 9 is a list of these diets and the experiments in which they were involved. Blending of the forage meal into the basal ration was carried out on a mechanically-driven rotary tumbler similar to devices for mixing paint or tumbling stones.

Since all crude forage diets were prepared on a weight-for-weight basis, little information on the amount of dilution of the basal ration can be concluded from the percentage composition values given in Table 9. Therefore, volume measurements were made on 10 g samples of dried forage meal, crude forage diet, and cellulose powder. The results are given in Table 10. From this data it may be seen that dried forages are twice as bulky as the control diet. When crude forage diets are involved, the percentage volume of plant in the ration is greater than the percentage by weight.

TABLE 9

## Crude Forage Diets

Diet no. <sup>a</sup>	Forage (by weight)	Forage sample <sup>b</sup>	Forage sample no. <sup>c</sup>	Experiment involved
1 <sup>d</sup>	0	---	---	all
Cf34	15%	3G Ladino	3	Reproduction Trial I
Cf35	30%	3G Ladino	3	Reproduction Trial I, Ova Collection Experiment, Vaginal Smear Experiment I
Cf36	60%	3G Ladino	3	Reproduction Trial I
Cf37	30%	1G Ladino	8	Reproduction Trial II
Cf38	30%	2G Ladino	13	Reproduction Trial III, Vaginal Smear Experiment II
Cf39	15%	1G Ladino	8	Weanling Experiment I
Cf40	15%	1G Ladino	11	Weanling Experiment I
Cf41	15%	2G Ladino	12	Weanling Experiment I
Cf42	15%	2G Ladino	13	Weanling Experiment II
Cf43	15%	4G Alfalfa	25	Weanling Experiment II



TABLE 9 (continued)

Diet no. <sup>a</sup>	Forage (by weight)	Forage sample <sup>b</sup>	Forage sample no. <sup>c</sup>	Experiment involved
Cf44	15%	Timothy grass	31	Weanling Experiment II
Cf45	15%	1G Ladino	17	Weanling Experiment III
Cf46	15%	1G Ladino	20	Weanling Experiment III
Cf47	15%	2G Ladino	22	Weanling Experiment III
Cf48	20%	1G Ladino	20	Weanling Experiment IV
Cf49	30%	1G Ladino	15	Transplant Experiment, Digestibility Study
Cf50	30%	2G Ladino	21	Digestibility Study

<sup>a</sup> Cf indicates crude forage.

<sup>b</sup> 1G = first growth; 2G = second growth; 3G = Third growth; 4G = Fourth growth.

<sup>c</sup> Further description of these samples is given in Tables 2, 3, and 4.

<sup>d</sup> Diet 1 = Control ration.

TABLE 10  
Approximate Densities of Various Diet Materials

Material	cc/g	Relative difference in volume <sup>a</sup>
Ladino clover	3.1	1.9
Alfalfa hay	3.5	2.2
Timothy grass	3.9	2.4
Cellulose powder	3.9	2.4
Control ration (Diet 1)	1.6	---
15% Ladino Diet	1.9	1.2
30% Ladino Diet	2.2	1.4
60% Ladino Diet	2.8	1.8

<sup>a</sup> Ratio of substance to control ration.

Other diets were used which do not fit into categories already characterized. These are explained in Table 11. The cellulose diets were compounded from chemically-prepared Whatman ashless powder (shown in figure 10). The source of the Ladino residue in diet 76 is given in figure 8. The Ladino residue in diet 77 resulted from an acetone Soxhlet extraction of forage sample 12. Diethylstilbestrol (DES) was dissolved in corn oil and diluted to the desired concentration. One milliliter of corn oil-DES solution was added to each 100 g of diet prepared. The

coumestrol for diets 80 and 81 was supplied by Dr. E. M. Bickoff, USDA Regional Laboratory, Albany, California. It was dissolved in acetone-alcohol (2:1) and added to basal ration in solution. The solvent was then removed from the diet under vacuum.

TABLE 11  
Other Diets

Diet no.	Description	Experiments involved
1	Purina Laboratory Chow (control ration)	all
74	15% Cellulose by weight	Weanling Experiment II, Weanling Experiment III, Transplant Experiment
75	30% Cellulose by weight	Transplant Experiment
76	20% Extracted Ladino Residue	Weanling Experiment IV
77	30% Extracted Ladino Residue	Vaginal Smear Experiment II
78	0.2 $\mu$ g diethylstilbestrol (DES) per gram of ration	Transplant Experiment
79	0.015 $\mu$ g DES per gram of ration	Interaction Experiment II
80	60 $\mu$ g coumestrol per gram of ration	Interaction Experiment II
81	0.015 $\mu$ g DES and 60 g coumes- trol per gram of ration	Interaction Experiment II

The major nutrient content of diets prepared from crude forages and their extracted residues was analyzed. Proximate analyses done by the Biochemistry Department provided the data shown in Table 12. Included for comparison are results of analysis of the control ration (diet 1). It

is apparent that no great differences in major nutrient content exist between the various crude forage diets and the control diet. These data do not provide information on the differences in the digestibility of the major nutrients. This was the object of a digestion study, the results of which are recorded in the Appendix.

TABLE 12  
Proximate Analysis of Certain Diets<sup>a</sup>

Ration	Ash	Fat	Fiber	Protein	N.F.E. <sup>b</sup>
Diet 1	7.40	5.44	5.09	26.43	55.64
Diet 77	8.87	4.55	9.45	26.10	51.03
Diet Cf38	7.36	5.10	9.12	24.95	53.47
Diet Cf49	8.94	5.18	6.96	27.47	51.44
Diet Cf50	9.27	5.14	7.44	26.44	51.71

<sup>a</sup> Results are in percentage dry matter.

<sup>b</sup> N.F.E. = nitrogen free extract.

Diets B2, B3, B4, and B5 were fed as dry pellets. All other diets were fed in petri dish bottoms as a wet paste. This procedure was decided upon for the following reasons:

1. The animals tend to scatter dry powdered diets, and much waste results.
2. Dry diets also necessitate the use of special feeding dishes which, for the purposes of these experiments, did not work well.
3. The pelleting procedure was too laborious and too

much time was required to prepare sufficient quantities of diet.

The wet paste was prepared at each feeding by mixing tap water with the diet until a thick, coalescent mash resulted. This was compressed with a spatula onto the bottom of the feeding dish. The animals would readily accept the ration in this form unless, as in the case of certain extract diets, unpalatable substances were present. The animals could scatter very little of the diet because of its sticky consistency. In view of a recent report by Keane et al. (1962) on the addition of water to purified diets and its effect upon growth, there may be other unexplained advantages in this method.

In the bioassay of estrogen-like substances in forages, extracts are usually administered in a fashion that will permit comparison of a uterine response with a standard dose-response curve for one or more specific compounds [Robinson (1949, Biggers and Curnow (1954), Cheng et al. (1953a), Pieterse and Andrews (1959), Kitts et al. (1959), Chury (1960)]. The amounts of material or activity in the forage is evaluated from a standard curve. This procedure implies that the biologically measured activity is equivalent to the activity of some one or two compounds. However, accurate evaluation of estrogen content cannot be made from such a comparison. This was well demonstrated by Bickoff et al. (1960), when it was shown that the estrogenic response obtained when a crude forage extract is assayed represents a

summation of effects of inhibitors and potentiators, together with that of the specific compounds in the extract. Payne (1953) found that variable results could also be obtained when different amounts of extract were tested. In view of these difficulties, few attempts were made to estimate the actual estrogenic content of the forages tested. A significant difference between the responses of controls and experimentals was the criterion most often used.

The Purina laboratory chow used in estrogen bioassays as the basal ration was checked for estrogenic activity. Negative results were obtained, and it was concluded that this material could be used for a negative control ration. Mr. P. L. Carey (personal communication, 1961) provided further information with regard to this subject. He guaranteed that this product would contain an activity less than that produced by seven parts per billion of diethylstilbestrol (DES). This is the lowest detectible amount of DES that gives a significant response in the method used by the Ralston Purina Company. Actually, this ration contains an activity much lower than seven parts per billion DES.

#### IV. EXPERIMENTAL PROCEDURE AND RESULTS

##### 1. ORAL BIOASSAY OF ESTROGENIC ACTIVITY

(a). With Immature Intact or Ovariectomized SWR/Jax Females. Ladino clover samples from the Piper-Newell field were tested for estrogenic content in 1960. Diets B2, B3, B4, and B5 were prepared from samples subjected to different harvesting procedures (Table 13) with the idea that various treatments might influence apparent estrogenic content. Bickoff et al. (1959) suggested that ovariectomized mice were less sensitive than immature intact females in forage estrogen determinations. To test this hypothesis, duplicate diets were administered to 45-day-old ovariectomized SWR/Jax mice, allowing a two-week postoperative period, and to 28-day-old immature females of the same strain.

Diets were offered ad libitum for four days, or until 75 g diet had been consumed per cage of five animals. At the conclusion of feeding, the animals were sacrificed by separation of the cervical vertebrae. The uteri were excised, trimmed of extraneous tissue, and placed in Bouin's fluid for two days. The uteri were then removed from the fixative, blotted between paper towels and weighed on a direct reading analytical balance.

All bioassay diets produced increased uterine weights when fed to intact females. However, castrated animals did

TABLE 13  
Bioassay of 1960 Ladino Clover With SWR/Jax Mice<sup>a</sup>

Diet	Harvesting treatment	Equivalent amt Ladino consumed <sup>b</sup> (g)	Uterine wt <sup>c</sup> (mg)	Uterus (% body wt)
Intact Animals				
1	--	0	27.1 ± 0.9	0.17
B2	none	3.0	36.3 ± 1.0	0.23
B3	none	3.0	34.0 ± 0.8	0.21
B4	sun dried	2.0	34.1 ± 0.7	0.20
B5	oven dried	1.0	31.4 ± 0.9	0.20
Castrated Animals				
1	--	0	13.8 ± 0.2	0.08
B3	none	3.0	10.8 ± 0.1	0.06
B4	sun dried	2.0	10.7 ± 0.2	0.05
B5	oven dried	1.0	12.2 ± 0.2	0.07

<sup>a</sup> Five animals per group.

<sup>b</sup> Calculated on dry matter basis per mouse.

<sup>c</sup> Mean ± SE.



did not respond, and uteri were reduced to levels below controls. No correlation between estrogenic activity and harvesting procedure was found. Uterine weight increase was directly related to the concentration of the extract consumed by intact females. Conversely, decrease in uterine weight was associated with the amount of the extract eaten by castrated females.

Forage sample 3, harvested in August, 1960, was tested for estrogenic activity with 21-day-old intact SWR/Jax females. Four control uteri averaged 8.8 mg. Five animals fed an acetone-extract of this sample had a uterine weight average of 9.5 mg. Since small numbers of animals were involved, the results were considered to be inconclusive. However, the slight increase in extract-fed animals could indicate that low levels of forage estrogen were present at this time.

(b). With Immature Intact CD-1 Females. All of the bioassays to follow were carried out with CD-1 mice. In order to facilitate reference to times when particular groups of animals were tested, each bioassay period was assigned a number. Table 14 is a list of these periods, and the specific dates are given for each interval.

Bioassays were attempted in 1961 on fresh Ladino samples from the Piper-Newell field. Ether extracts (bioassay diets) and water solubles remaining after ether extraction (experimental extract diets) were tested for estrogenic

activity using immature female CD-1 mice. Diets B6 through B12 and diets E54 through E60 were prepared from these samples. Further information pertaining to these diets is given in the Materials and Methods section.

TABLE 14  
Bioassay Periods With CD-1 Mice

Period no.	Dates	Length of feeding
1	5/14 to 5/20/61	6 days
2	5/25 to 5/31/61	6 days
3	6/23 to 6/29/61	6 days
4	7/6 to 7/12/61	6 days
5	8/16 to 8/22/61	6 days
6	8/21 to 8/26/61	5 days
7	10/18 to 10/25/61	7 days
8	12/7 to 12/13/61	6 days
9	3/1 to 3/7/62	6 days
10	6/8 to 6/14/62	6 days
11	6/27 to 7/3/62	6 days
12	7/20 to 7/26/62	6 days
13	7/25 to 7/31/62	6 days

Diets prepared from Ladino samples collected in May, 1961, were fed to 24-day-old females (Table 15). Three grams of diet per mouse per day was dispensed at 6 PM for six days. Five mice were tested on each diet, and each group was housed in a separate cage. The mice, at 30 days of age, were

sacrificed early in the morning following the last feeding. Uteri were excised and trimmed under a dissecting microscope. Uteri were immediately weighed in the fresh condition, on an analytical balance. Prior to weighing, the uteri were placed in small humidors containing a wad of cotton moistened with physiological saline solution. It was felt that this procedure would minimize weight loss due to dessication between extirpation and weighing.

TABLE 15  
Bioassay With 24-Day-Old CD-1 Females<sup>a</sup>

Diet	Bioassay period	Avg Uterine wt (mg)	Avg Body wt (g)	Uterus (% body wt)
1	1	17.3 $\pm$ 2.5 <sup>b</sup>	18.2	0.10
1	2	24.4	16.2	0.15
1	3	39.1	20.8	0.18
B6	1	31.1 $\pm$ 3.9 <sup>b</sup>	19.6	0.16
B7	2	19.1	16.6	0.12
B8	3	42.5	18.8	0.23
E54	1	33.8 $\pm$ 5.8 <sup>b</sup>	20.4	0.17
E55	2	17.7	16.4	0.11
E56	3	46.0	20.8	0.22

<sup>a</sup> Five animals per group

<sup>b</sup> Standard error

The 15 control uteri represented in Table 15, when pooled, had a value of 26.9  $\pm$  6.4 mg (mean  $\pm$  SE). By comparing this figure to individual groups of controls, it

can be seen that considerable deviation occurred between different bioassay periods. Therefore, results from experimentally fed animals can only be compared with control values determined for the same bioassay period. A significant uterine response was produced by diet B6 ( $P = .05$ ). The remainder of the groups fed experimental rations did not have uterine weights significantly different from controls. However, the variance in uterine weights for groups tested during bioassay periods 2 and 3 was sufficiently high so that small responses could easily have been concealed.

Fresh Ladino samples collected in May and June, 1961, were tested (Table 16). The bioassay animals were 21 days old at the start of the feeding period, and the procedure was the same as with 24-day-old females. All animals were 27 days of age when sacrificed.

Control uteri, when considered together, averaged  $30.0 \pm 5.4$  mg (mean  $\pm$  SE). As with 24-day-old animals, the variation in uterine weight within groups resulted in highly deceptive average values. No significant increases in uterine weight could be found in animals fed experimental rations. Diethylstilbestrol, at the levels used, did not produce a significant uterine response.

Since 24- and 21-day-old CD-1 females did not produce constant uterine weight averages, younger animals were tried. It was anticipated that less deviation would occur in 18-day-old animals. Therefore, females of this age were tested with diets prepared from Ladino clover and alfalfa (Table 17).

Three DES reference standards were included for comparison. The procedure was as before with 24- and 21-day-old animals. At the conclusion of the bioassay periods, the mice were 23 to 24 days of age.

TABLE 16  
Bioassay With 21-Day-Old CD-1 Females<sup>a</sup>

Diet	Bioassay period	Avg Uterine wt (mg)	Avg Body wt (g)	Uterus (% body wt)
1	4	23.8	17.8	0.13
1	4	45.5	18.4	0.25
1	5	20.6	17.8	0.12
B9	4	26.8	18.0	0.15
B10	4	28.8	16.6	0.17
B11	4	17.1	17.4	0.10
E57	4	38.3	18.0	0.21
E58	4	18.8	17.0	0.11
E59	4	15.1	16.8	0.09
DES-1 <sup>b</sup>	5	30.8	18.5	0.17
DES-2 <sup>c</sup>	5	32.3	20.0	0.16

<sup>a</sup> Five animals per group.

<sup>b</sup> DES-1 = 0.0025  $\mu$ g DES per gram diet l.

<sup>c</sup> DES-2 = 0.005  $\mu$ g DES per gram diet l.

Less deviation than before was found in animals tested in bioassay period 6. However, statistical analysis revealed no significant uterine weight differences during this period. In bioassay period 7, diet B14 administration

resulted in a significantly higher uterine weight average ( $P = .05$ ). None of the DES standards produced significant responses. All of the control uteri in Table 17, when consolidated, have a value of  $20.8 \pm 2.3$  mg (mean  $\pm$  SE).

TABLE 17  
Bioassay With 18-Day-Old CD-1 Females

Diet	Bioassay period	No. Animals	Avg Uterine wt (mg)	Avg Body wt (g)	Uterus (% body wt)
1	6	10	12.8	12.6	0.10
1	7	17	25.5	17.0	0.15
B10	6	5	14.0	13.0	0.11
B11	6	7	13.8	11.3	0.12
B12	6	5	14.8	14.4	0.10
B12	7	5	16.3	14.6	0.11
B13	7	8	30.0	16.9	0.18
B14	7	8	39.0	17.5	0.22
E60	6	6	9.9	12.4	0.08
DES-1 <sup>a</sup>	6	5	12.2	12.5	0.10
DES-2 <sup>b</sup>	6	5	13.0	13.5	0.10
DES-2 <sup>b</sup>	7	10	28.3	15.7	0.18
DES-3 <sup>c</sup>	6	5	14.9	12.6	0.12

<sup>a</sup> DES-1 =  $0.0025 \mu\text{g}$  DES per gram diet l.

<sup>b</sup> DES-2 =  $0.005 \mu\text{g}$  DES per gram diet l.

<sup>c</sup> DES-3 =  $0.025 \mu\text{g}$  DES per gram diet l.

Statistical analysis of the data from 24-, 21-, and 18-day-old intact females showed that there were significant differences between the uterine responses of different age groups. With regular bioassay diets (B) and experimental extract diets (E), significant differences ( $P = .05$ ) were found in the uterine weights when all data were combined. No such effects were found in controls or females fed DES in the control ration. This seems to indicate that biologically active substances were present in forage-extract diets. However, very little can be said as to the effects of specific diets except in the few cases where the response was great.

Uteri from animals used in the bioassay of fresh Ladino clover samples were preserved in Bouin's fluid following fresh weight determinations. After 72 hours in the fixative, these uteri were blotted between paper towels and weighed again. A product moment correlation was then made on data obtained from fresh and fixed uteri. The correlation coefficient between fixed uteri and fresh uteri was for controls = 0.99, B diets = 0.98, and E diets = 0.99.

It was concluded that fresh uterine weights have the same relative value as fixed uterine weights.

(c). Comparison of Immature Intact, Ovariectomized and Hypophysectomized CD-1 Females. The nature of the bioassay results with intact CD-1 mice suggested that prepuberal ovaries might be secreting estrogen in variable amounts. This, of course,

would often mask uterine responses to weak estrogens such as those occurring in legumes. Therefore, it was felt that ovariectomized or hypophysectomized immature mice might be useful in studying this possibility.

CD-1 females, 9 to 10 g, were weaned and prepared at 18 days of age by the Charles River Breeders. Intact, ovariectomized and hypophysectomized animals were supplied one day later. Three groups of each kind of animal were selected at random. Diet 1 and a DES reference standard were administered to a group of each type of animal. Diet B13, tested in a previous period, was suspected of containing estrogenic activity and was included as the experimental ration. The feeding schedule is shown in Table 18.

TABLE 18  
Feeding Schedule For Bioassay Period 8

Age	g Diet/mouse	
	8 AM	6 PM
Day 19	--	2.5
Day 20	--	2.5
Day 21	--	2.5
Day 22	1.5	2.5
Day 23	1.5	2.5
Day 24	1.5	2.5
Day 25	Sacrificed in early AM	



Hypophysectomized animals were given 5% glucose in the drinking water. In spite of this precaution, severe mortality occurred in these animals. Before the completion of the feeding period, 67% of the starting number had succumbed. The surviving animals consumed very little food during the bioassay period. This fact makes it impossible to compare results from these animals with data from ovariectomized and intact animals.

All of the animals were sacrificed at 25 days of age. Fresh uteri were weighed at this time, and characteristics of the vagina were noted. Vaginal patency was considered as a complete perforation of the vaginal membrane. This would be analogous to stage 5 in the method used by Hartman, Littrell and Tom (1946). (See Table 19.)

Intact females had highly variable uterine weights, as can be seen from the standard errors in Table 19. Neither diet B13 nor DES produced uterine responses significantly different from controls. Vaginal patency occurred in all groups of intact females. The largest response in this respect was found in animals fed diet B13.

Ovariectomized females on control ration had very low uterine weights and no vaginal patency. This result strongly suggested that normal CD-1 females secrete endogenous estrogen even at this very early age. DES at 0.01  $\mu$ g per gram of diet 1 caused a significant increase in uterine weight ( $P = .05$ ). No vaginal opening resulted as a consequence of DES application, however. The most startling

TABLE 19  
 Bioassay Comparing Intact, Ovariectomized and  
 Hypophysectomized 19-Day-Old CD-1 Females<sup>a</sup>

Diet	No. animals	Avg Uterine wt (mg)	Avg Body wt (g)	Uterus (% body wt)	Vaginal patency
Intact					
1	7	33.3 $\pm$ 10.2 <sup>c</sup>	17.1	0.19	50%
B13	6	46.0 $\pm$ 13.4	17.0	0.27	100%
DES-4 <sup>b</sup>	12	27.2 $\pm$ 6.4	17.8	0.15	75%
Ovariectomized					
1	9	6.2 $\pm$ 0.03 <sup>c</sup>	17.3	0.04	0%
B13	7	35.1 $\pm$ 8.7	16.8	0.21	100%
DES-4 <sup>b</sup>	7	11.1 $\pm$ 0.8	17.1	0.06	0%
Hypophysectomized					
1	2	5.4	--	--	--
B13	2	20.8	--	--	--
DES-4 <sup>b</sup>	3	5.0	--	--	--

<sup>a</sup> Bioassay period 8.

<sup>b</sup> DES-4 = 0.01  $\mu$ g DES per gram diet 1.

<sup>c</sup> Standard error.

effect was that produced by diet B13 feeding. A sixfold increase in uterine weight was found, and this was highly significant ( $P = .01$ ). Also, complete vaginal opening occurred in all animals fed diet B13.

Since such few numbers of hypophysectomized animals survived, and in view of their poor diet consumption, results from these animals probably mean very little. Diet B13 did appear to cause increased uterine weights in two animals.

(d). With Ovariectomized 19-Day-Old CD-1 Females.

Diets used in Extract Reproduction Experiment II were tested for estrogenic activity. Ovariectomized 19-day-old females, 9 to 10 g, were chosen as test animals because of the findings shown in Table 19. The procedure and feeding schedule was the same as in bioassay period 8. The results are given in Table 20. Also included are data on two DES reference standards. Diet B15 was considered to contain the total activity in the sample from which diets E67 and E68 were prepared.

Diets E68 and B15 produced significant increases in uterine weight as compared to controls ( $P = .05$ ). Since animals on Diet E68 exhibited fewer vaginal responses, this ration was thought to contain less activity than diet B15. The results obtained from animals fed diet E67 are difficult to explain because of decreased body weight at the end of assay. The diet consumption data in Table 21 may help to clarify this point.

Except for diet E67, all groups consumed similar amounts of the various diets during bioassay period 9. It

would seem that two possible explanations can be offered for the low body weights of animals fed diet E67. The diet may have contained materials which caused metabolic effects within animals, or it is conceivable that the ration was unpalatable to the mice. Had this diet been eaten in amounts comparable to the other diets, a greater estrogenic response might have been obtained. As to whether this diet contained significant estrogenic activity, considerable doubt still remains. It is interesting to note that the uterine weight average for animals fed diet E67 was below values for diets E68 and B15. However, because body weights were low, the uterus as percentage of body weight was increased to a figure comparable to values obtained with estrogenic diets.

Table 20

Bioassay of Extract Reproduction Experiment II Diets<sup>a</sup>

Diet	Avg Uterine wt (mg)	Avg Body wt (g)	Uterus (% body wt)	Vaginal patency
1	7.8	16.8	0.05	0
E65	7.8	16.9	0.05	0
E66	7.8	17.0	0.05	0
E67	9.3	12.9 <sup>b</sup>	0.07	0
E68	10.2	17.0	0.06	33%
B15	12.7	17.8	0.07	100%
DES-5 <sup>c</sup>	24.7	17.0	0.15	50%
DES-6 <sup>d</sup>	59.9	17.3	0.35	100%

<sup>a</sup> Bioassay period 9; six animals per group.

<sup>b</sup> Significantly lower than controls ( $P < .01$ ).

<sup>c</sup> DES-5 = 0.03  $\mu$ g DES per gram diet 1.

<sup>d</sup> DES-6 = 0.06  $\mu$ g DES per gram diet 1.

TABLE 21  
Diet Consumption of Ovariectomized Females  
During Bioassay Period 9

Diet <sup>b</sup>	Total amt fed (g)	Refuse (g)	Consumption (g)
1	117	8	109
E65	117	12	105
E66	117	14	103
E67	117	51	66 <sup>a</sup>
E68	117	11	106
B15	117	11	106
DES-5	117	8	109
DES-6	117	8	109

<sup>a</sup> Significantly lower ( $P = .01$ ).

<sup>b</sup> Six animals per group.

Ladino clover and alfalfa samples collected in 1962 were tested for estrogenic activity. Ovariectomized 19-day-old females were used according to the procedure previously employed in bioassay periods 8 and 9. Both positive and negative controls were used. Negative controls were fed diet 1. Positive controls were fed diet B13 which was known to be estrogenic. The latter ration had been stored for seven months at 4 C in a sealed bottle. Diet B16 was prepared from an alfalfa sample, previously shown to be estrogenic, which had been stored at room temperature for ten months. The remainder of the bioassays diets were from 1962 samples. The

TABLE 22

Bioassay of 1962 Legume Samples<sup>a</sup>

Diet	Bioassay period	Avg Uterine wt (mg)	Avg Body wt (g)	Uterus (% body wt)	Vaginal patency
1	10	7.8	17.1	0.05	0
1	11	6.9	16.7	0.04	0
1	13	7.2	16.8	0.04	0
B13	10	22.8	16.7	0.14	100%
B16	11	11.0	16.8	0.07	50%
B17	10	7.5	17.3	0.04	0
B18	10	8.6	16.9	0.05	0
B19	10	9.3	16.8	0.06	0
B20	10	8.3	17.2	0.05	17%
B21	10	8.2	17.5	0.05	0
B22	10	7.7	17.3	0.04	0
B23	10	7.4	16.9	0.04	0
B24	10	8.8	17.1	0.05	0
B25	11	7.9	16.7	0.05	0
B26	11	9.7	16.7	0.06	0
B27	12	10.3	15.6	0.07	0
B28	13	8.5	16.6	0.05	17%
B29	13	6.2	17.2	0.04	0
B30	13	7.6	16.3	0.05	0
B31	13	7.1	16.4	0.04	0
B32	13	8.9	15.3	0.06	0
B33	13	6.7	16.4	0.04	0
Coumestrol <sup>b</sup>	11	26.4	18.1	0.15	100%

<sup>a</sup> Six animals per group.<sup>b</sup> 120  $\mu$ g coumestrol per gram diet 1.

results are shown in Table 22, and a coumestrol diet is included as a reference standard.

Diets B13 and B16 produced significant increases in uterine weights and vaginal openings ( $P = .05$ ). None of the other bioassay diets showed estrogenic activity at the level of significance. Statistical analysis of the data from 1962 samples was carried out. Uterine weights of negative controls were combined as were those from animals fed extracts of Ladino samples. A slight average increase (0.6 mg) was then found in the uterine weights of animals fed on bioassay diets prepared from Ladino samples. A larger increase (2 mg) was found to result with alfalfa bioassay diets. This undoubtedly means that very small estrogenic effects were experienced by the animals when fed most of these bioassay diets.

## 2. REPRODUCTION STUDIES

(a). Reproduction Trial I. Thirty-two mature (three months old) SWR/Jax females weighing 18 to 22 g were divided into four equal groups and housed four per cage. The control group was fed diet 1 and the remaining groups were maintained on diets Cf34, Cf35, and Cf36. These diets are explained in Table 9. All diets were administered at 5 g per mouse per day during a preliminary feeding period of seven days. Males of the same age and strain were then introduced, two per cage, and allowed cohabitation with females for 22 days. All groups were maintained on their respective prefeeding rations. At the conclusion of this phase, the males were removed, and females were continued on their respective diets through a theoretical gestation period (i.e., for an additional 22 days). Experimental females were regrouped with their respective males upon termination of the forage-feeding period, and diet 1 was substituted at that time for the clover diets.

No pregnancies occurred in females while treated with diets Cf34 and Cf35. Diet Cf36 proved to be too unpalatable to the mice during seven days of prefeeding, and 57% pregnancies subsequently resulted when diet 1 was substituted. Return to a fertile condition required about five days of readjustment following the cessation of feeding diet Cf36. In all cases of Ladino feeding, an initial loss in body weight occurred which was proportional to the amount of



clover in the diet (21% loss on Cf36, 16% on Cf35, and 4% on Cf34). A graph of the average body weights of females is shown in figure 11. Weight gains began to appear after 18 days of feeding, indicating an adaptation to the crude forage diets. Feeding of the basal diet, after 50 days of Cf34 or Cf35, allowed 75% and 37% pregnancies, respectively. However, a four to five day readjustment period was necessary before fertility was regained, similar to results obtained with animals briefly treated with diet Cf36. Littering results are given in Table 23.

TABLE 23  
Fertility of SWR/Jax Females  
(Reproduction Trial I)

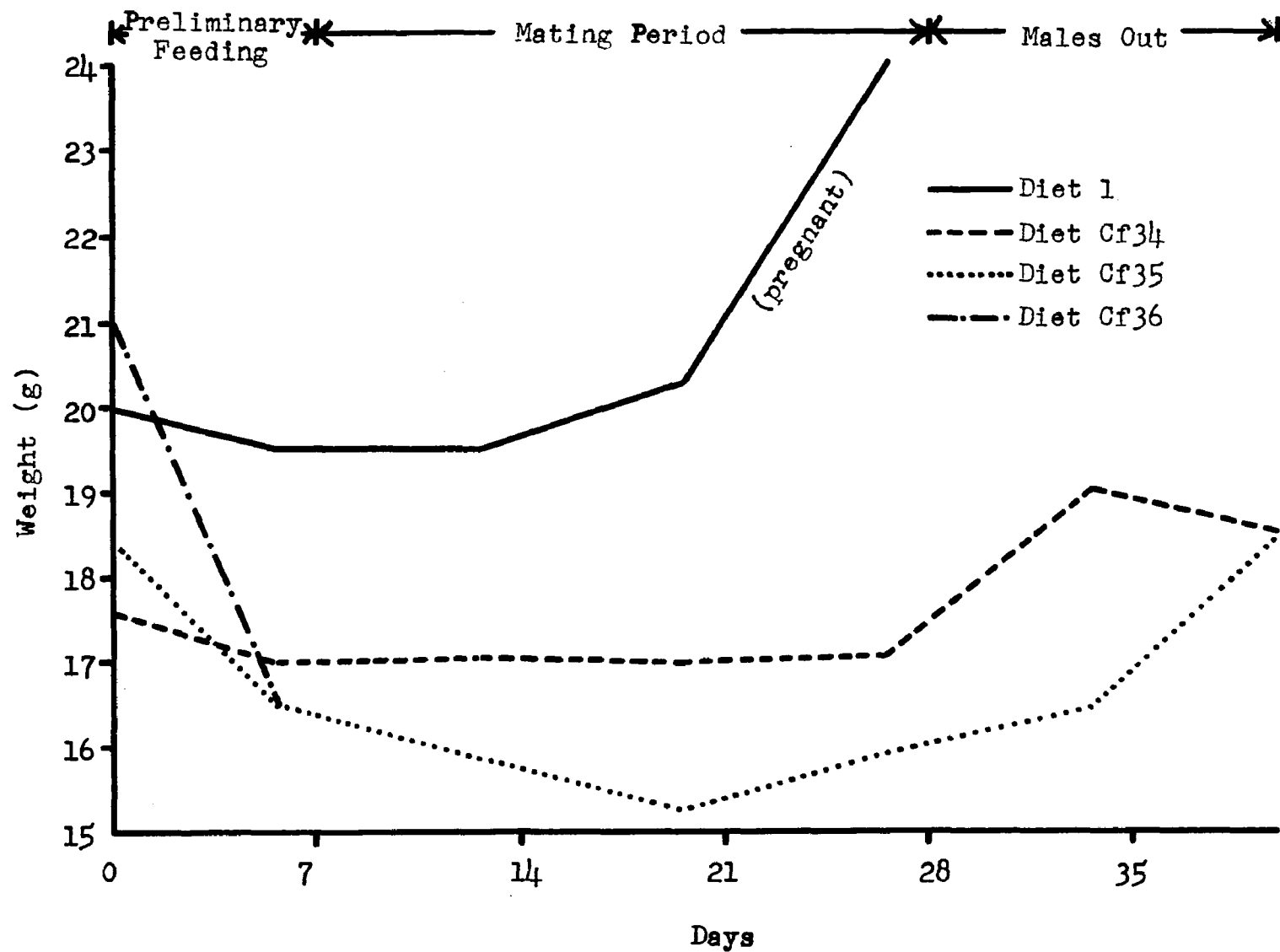
Previous dietary regimen <sup>a</sup>	No. females	No. litters	Avg no. young weaned
Diet 1	8	4	6
Diet Cf34	8	6	4
Diet Cf35	8	3	4
Diet Cf36	7 <sup>b</sup>	4	5

<sup>a</sup> Pregnancies in all groups occurred during treatment with diet 1.

<sup>b</sup> One female died during the prefeeding period.

(b). Reproduction Trial II. In order to explore the preceding results in more detail, males were tested separately. Since SWR/Jax controls were not 100% fertile in the previous experiment, the CD-1 strain was selected, because it was alleged to have a higher fertility index and larger litters.

Fig. 11. Average Weight of Females - Reproduction Trial I.



Sixteen mature CD-1 males, ranging in age from three to seven months, were allotted at random into two equal groups. Each male was housed in an individual cage. One group was maintained on diet 1 and the other on diet Cf37. Seven grams of diet per day per mouse was the level fed, and this proved to more than exceed actual consumption.

After a five-day prefeeding period, mating trials were carried out with females of unproven fertility. Between 4-6 PM, vaginal smears were taken from mature CD-1 females by means of the lavage technique (Snell, 1941). Individuals showing early estrous smears were caged with males, one female to one male, from 7 PM to 6 AM. Positive matings were determined the following morning. The presence of a copulation plug in the vagina or sperm in a vaginal smear indicated that mating had occurred. Mating trials were attempted with each male until a positive mating resulted or until four different females had been proffered. The results are shown in Table 24.

TABLE 24

Mating Results With CD-1 Males  
(Reproduction Trial II)

	No. males	No. positive matings	No. trials	Matings per trial	No. fertile matings
Diet 1	8	8	12	0.67	7
Diet Cf37	8	7	13	0.54	6

At the conclusion of the previously described mating trials, the eight animals formerly receiving diet 1 were

prefed for five days on diet Cf37. Mating trials were then carried out as before. All mated positively with 0.62 matings per trial, and seven matings were fertile.

Weight records were difficult to interpret because the period of treatment with a particular ration varied according to the mating behavior of individual animals. The eight males fed control ration maintained their weights at about 42.5 g. Animals receiving Cf37 lost about 1.5 g per week. Since Ladino feeding did not seem to impair male CD-1 fertility under the conditions of this study, the slight differences in weight records were not regarded as being significant.

(c). Reproduction Trial III. The results in Reproduction Trial II suggested that the infertility observed in Reproduction Trial I could not be attributed to males. Therefore, female CD-1 mice were treated as before and tested with untreated males of proven fertility. Vaginal smears were taken daily on two-month-old females for 12 days to check for regularity of estrous activity. Diet Cf38 was administered to eight of these animals in the morning and evening at 6 g per mouse per day. After a seven-day prefeeding period, estrous females, as determined by vaginal smears, were housed overnight in a separate cage with males. Positive matings were determined as described in Reproduction Trial II.

The first estrous smear, after the prefeeding period, occurred 18 days following the start of the experiment. Six animals had estruses by day 28. The majority of animals did not mate immediately upon reappearance of vaginal estrus,

however. Estrous smears had not reappeared in the remaining two females by day 40, and mating trials were terminated at this time. Six animals mated, and all matings were fertile. There were 0.46 matings per trial, and 9.34 young per litter (range = 8 to 11). Body weight records were inconsistent and could not be evaluated. Supplementary information on the effects of diet Cf38 are reported in Vaginal Smear Experiment II.

(d). Extract Reproduction Experiment I. The preparation of diets for this experiment is explained in the Materials and Methods section, and the extraction scheme is shown in figure 5. Fifty-four CD-1 females one month of age were obtained. These were preconditioned to cages, and vaginal smears were taken to determine estrous activity. Twelve were selected as controls and fed diet 1 throughout. Thirty-two were divided into four equal groups, all of which were eventually fed experimental extract diets. The various diets are explained in Table 8. In all cases, four females were allowed per cage, and cages were distributed in a random block design in the mouse room.

All diets were prefed for seven days, followed by an experimental feeding and mating period of 22 days. Mating trials were carried out as described in Reproduction Trial III. Vaginal smears were taken after mating to check for reappearance of estruses and were terminated when pregnancy was visibly apparent (14 to 15 days post coitum).

A total of 6 g of diet per mouse was administered daily in two feeding periods: 8 AM to 6 PM and 6 PM to 8 AM. Two grams per mouse were fed during the former interval, and 4 g per mouse during the latter. The females were two months old at the start of the mating period. Body weights were not determined in order that handling of animals would be kept at a minimum. The number of young per litter was determined at birth.

There were no significant differences in the size of litters, number of positive matings, and number of pregnancies (Table 25). One pseudopregnancy resulted from diet E62 treatment, and another was observed with an animal receiving diet E64. The number of matings per trial was similar in animals fed diets 1, E62, and E64. Mating behavior for females on diet E61 was low, while females on diet E63 mated readily.

TABLE 25

Mating and Fertility Data  
(Extract Reproduction Experiment I)

Diet	No. Animals	No. Positive matings	No. Matings per trial	No. Preg- nancies	No. Pseudo- preg.	Litter size <sup>a</sup>
1	12	11	0.58	11	0	9.4 $\pm$ 0.74
E61	8	8	0.42	8	0	10.1 $\pm$ 1.18
E62	8	9	0.60	8	1	10.3 $\pm$ 0.81
E63	8	8	0.80	8	0	11.1 $\pm$ 0.61
E64	8	8	0.57	7	1	9.9 $\pm$ 0.93

<sup>a</sup> Arithmetic Mean  $\pm$  SE.

Further information on mating behavior is provided in Table 26. The numerical values for first estrus and first mating have little meaning because estrous cycles were of varying lengths and were not synchronized at the beginning of the mating period. However, the longer intervals between first estrus and first mating explain some of the reduced mating behavior observed.

TABLE 26  
Average Time (Days) of First Vaginal Estrus  
and First Mating<sup>a</sup>

(Extract Reproduction Experiment I)

Diet	No. animals	First estrus	First mating	Interval between
1	11	7.18	8.64	1.46
E61	8	4.75	8.12	3.37
E62	8	5.00	6.50	1.50
E63	8	9.25	9.50	0.25
E64	7	3.57	4.57	1.00

<sup>a</sup> Day 1 = Start of mating trials.

(e). Extract Reproduction Experiment II. The preparation of diets for this experiment is covered in the Materials and Methods section, and data on the estrogen content of the diets is shown in Table 20. The diets are explained in more detail in Table 8. Forty CD-1 females three months old were selected for cyclic estrous activity. All were known to be fertile. These animals were divided at random into five equal groups. Four animals were housed per cage, and the cages were distributed in the mouse room according to a random block design.

The feeding procedure and diet allotment was the same as in Extract Reproduction Experiment I. Five days were allowed for adjustment at the onset of experimental feeding, and this was followed by a 23-day mating period. Mating trials were performed as before with males of proven fertility. Vaginal smears were taken on mated animals in order to determine pseudopregnancies. This procedure was discontinued upon the appearance of physical manifestations of pregnancy.

All pregnant females were sacrificed 15 days post coitum. At autopsy viable embryos were counted and weighed fresh on a direct reading analytical balance. Non-viable embryos were also accounted for at this time. Body weights were recorded prior to experimental feeding and at the time of autopsy. Table 27 shows the weight relationship between the females and their offspring.

TABLE 27

Average Weights (g) of Mothers and Embryos  
(Extract Reproduction Experiment II)

Diet	Females before mating	Mother at day 15 of gestation	Wt gain	Viable <sup>a</sup> embryos	<u>Wt Mother</u> <u>Wt Litter</u>
1	36.4	47.4	11.0	0.26	16.7
E65	36.4	46.5	10.1	0.25	16.9
E66	37.5	48.7	11.2	0.28	15.0
E67	35.5	44.5	9.0	0.26	17.0
E68	34.2	44.0	9.8	0.27	16.7

<sup>a</sup> Mean wt per embryo.



Data pertaining to mating behavior and fertility are shown in Tables 28 and 29. There were no significant differences between the numbers of embryos, pregnancies, or matings per trial. One pseudopregnancy resulted from diet E66 feeding, and three were produced from diet E67 treatment. The number of resorptions increased in all cases of experimental extract feeding. Animals receiving diet E67 had more than twice as many resorptions as animals on control ration. Diet E68 feeding also resulted in an increased number of resorptions.

Feed consumption was measured daily, before and during the experimental feeding period. A graph of average values is shown in figure 12. Periods 1 and 2 occurred just prior to experimental feeding. It can be seen that feed consumption dropped considerably during the first eight days of extract diet feeding. By period 5, after eight days of experimental feeding, consumption had returned to normal. The explanation for the decrease in diet consumption is not readily apparent, but it seems to be correlated with the amount of extracted material in the diet. Palatability may be involved. However, this possibility does not seem to be consistent with the observation that all animals readily accepted extract diets when they were first offered.

Fig. 12. Average Diet Consumption (g) During Consecutive Four Day Periods of Extract Reproduction Experiment II.

1	Diet 1	3.75
2		3.66
3	Diet 1	3.59
4		3.35
5		4.08
1	Diet 1	4.03
2		3.69
3	Diet E65	2.69
4		2.65
5		3.49
1	Diet 1	3.88
2		4.00
3	Diet E66	3.34
4		2.92
5		3.72
1	Diet 1	3.97
2		4.16
3	Diet E67	3.13
4		3.27
5		4.36
1	Diet 1	4.78
2		4.53
3	Diet E68	4.00
4		4.07
5		4.48

[ 1=Smear Period 3=Prefeed Period 5=Mating Period  
2=Smear Period 4=Prefeed Period ]

TABLE 28

Mating and Fertility Data<sup>a</sup>  
(Extract Reproduction Experiment II)

Diet	No. Positive matings	No. Matings per trial	No. Preg- nancies	No. Psuedo- preg.	No. Viable <sup>b</sup> embryos	Mean no. resorp- tions
1	8	0.73	8	0	10.9 $\pm$ 0.61	0.8
E65	8	0.80	8	0	11.0 $\pm$ 1.05	1.0
E66	9	0.69	8	1	11.6 $\pm$ 0.87	1.1
E67	11	0.79	8	3	10.1 $\pm$ 1.29	2.1
E68	8	0.80	8	0	9.8 $\pm$ 0.96	1.5

<sup>a</sup> Eight animals per group.

<sup>b</sup> Mean  $\pm$  SE at day 15 of gestation.

TABLE 29

Average Time (Days) of First Vaginal Estrus  
and First Mating<sup>a</sup>

(Extract Reproduction Experiment II)

Diet	First estrus	First mating	Interval between
1	3.25	4.12	0.87
E65	4.87	5.25	0.38
E66	2.62	4.50	1.88
E67	3.50	5.25	1.75
E68	3.75	4.00	0.25

<sup>a</sup> Eight animals per group. Day 1 = start of mating period.

### 3. STUDIES WITH FEMALES FROM WEANING AGE

(a). Weanling Experiment I. Forty-three immature female mice, weaned at 18 days of age, were divided into four groups. One group received diet 1, and the other three groups were fed diets Cf39, Cf40 and Cf41, respectively. These diets are explained in Table 9. Feeding commenced at weaning and continued for 27 days. Six grams per mouse per day was administered at two feedings each day, 8 AM and 6 PM. The animals were inspected daily for vaginal opening, and daily vaginal smears were taken thereafter until the animals were 45 days old. Vaginal introitus, in this experiment, was considered to be any form of perforation of the vaginal membrane. Body weights were recorded on alternate days throughout.

Vaginal smears furnished evidence of estrous activity (Table 30). Controls and animals raised on diet Cf41 did not appear to be adversely affected in this respect. In fact, females treated with Cf41 exhibited a somewhat higher estrous index (17) than did controls (14). Diet Cf39 definitely inhibited the appearance of vaginal estrus as estimated by estrous index (5) and the number of animals showing estrous smears by day 45. Feeding diet Cf40 did not appear to reduce the estrous index, but did result in fewer estruses than in controls.

No significant differences between groups in regard to the appearance of vaginal introitus were observed. Table

31 contains this data together with relative growth rates (K) for the log-growth phase (18 to 28 days of age). Brody (1945) described the term (K) as being similar to the velocity constant of the chemist, provided that it is applied to periods of early growth when the principle of mass action is applicable.

TABLE 30  
Vaginal Estrous Activity  
(Weanling Experiment I)

Diet	No. Animals	No. Smears	No. Estrous smears	No. Estruses	Estrous index <sup>a</sup>	% of animals showing estrus by day 45
1	14	185	26	19	14	100
Cf39	9	125	6	5	5	44
Cf40	10	144	19	11	13	70
Cf41	10	121	21	16	17	100

$$^a \text{ Index} = \frac{\text{No. estrous smears}}{\text{No. smears}} \times 100$$

Body weights were not significantly different between groups because of high variability within groups. However, it should be noted that controls (diet 1) gained more weight at a faster rate than any of the other groups. Animals on diets Cf40 and Cf41 gained at somewhat slower rates and attained final weights (day 45) slightly less than controls. Females raised on diet Cf39 gained weight at a slow rate (K = .027) and reached a final weight much lower than any of the other groups.

TABLE 31  
Vaginal Opening and Relative Growth Rate (K)  
(Weanling Experiment I)

Diet	Vaginal introitus <sup>a</sup> (days of age)	K <sup>b</sup>
1	31 $\pm$ 0.9	.033
Cf39	31 $\pm$ 1.1	.027
Cf40	30 $\pm$ 1.9	.029
Cf41	32 $\pm$ 0.8	.032

<sup>a</sup> Arithmetic mean  $\pm$  SE.

<sup>b</sup>  $K = \frac{\log \text{weight}_2 - \log \text{weight}_1}{\text{Age}_2 - \text{Age}_1} = \log \text{ g/day.}$

(b). Weanling Experiment II. Forty-five females, weaned at 18 days, were allotted into five groups. One group of five control animals received diet 1. The other four groups, ten animals in each, were fed diets Cf42, Cf43, Cf44 and 74, respectively. These diets are listed in Tables 9 and 11. The experimental procedure was the same as that in Weanling Experiment I, except for differences in the levels of diet fed. The feeding schedule is shown in Table 32. All animals were sacrificed at 45 days of age, and the fresh weights of both ovaries and both adrenals were determined at this time on a direct reading analytical balance.

Table 33 contains data related to estrous activity. Since only five animals were used as controls, the estrous index for diet 1 may be unreliable. Animals on diets Cf42 and Cf44 had an index which appeared to be quite high. Data

TABLE 32  
Feeding Schedule  
(Weanling Experiment II)

g Diet/mouse		
Age	8 AM feeding	6 PM feeding
18-19 days	---	2.0
20-27 days	1.5	2.5
28-34 days	2.0	3.0
35-45 days	2.0	4.0

TABLE 33  
Vaginal Estrous Activity  
(Weanling Experiment II)

Diet	No. Animals	No. Smears	No. Estrous smears	No. Estruses	Estrous index <sup>a</sup>	% Animals showing an estrus
1	5	98	16	12	16	100
Cf42	10	155	35	25	23	100
Cf43	10	176	24	16	14	80
Cf44	10	95	18	15	19	80
74	10	157	16	12	10	90

$$^a \text{ Index} = \frac{\text{No. Estrous smears}}{\text{No. Smears}} \times 100$$

in Table 34 help to explain the high index associated with Cf44 but not Cf42. Cf44-fed animals had a significantly later vaginal opening than did controls ( $P < .05$ ). Consequently, fewer smears were taken on these females, and estruses appeared at about the same time as occurred in other groups. Diet Cf43 was known to be estrogenic (see Part 1 of this section). No apparent effect on estrous activity could be deduced from Cf43 administration. However, vaginal introitus occurred in animals on this diet at earlier ages than with controls or animals fed cellulose (diet 74).

TABLE 34  
Vaginal Opening and Relative Growth Rate (K)  
(Weanling Experiment II)

Diet	Vaginal introitus <sup>a</sup> (days of age)	K <sup>b</sup>
1	30 $\pm$ 1.1	.031
Cf42	32 $\pm$ 0.7	.032
Cf43	28 $\pm$ 0.7	.027
Cf44	35 $\pm$ 1.2	.025
74	30 $\pm$ 1.1	.028

<sup>a</sup> Mean  $\pm$  SE

<sup>b</sup> K was computed for log-growth phase (18 to 28 days of age). See Table 31 for formula.

As in Weanling Experiment I, no significant differences occurred between groups in respect to body weights. However, there was high variability in weights within groups. Controls (diet 1) had the highest weights throughout. Animals



on diets Cf42, Cf43 and 74 gained at variable rates, as shown by the values for K in Table 34. Females fed diet Cf44 gained at the slowest rate and reached a final weight much lower than controls. The values for average body weight at day 45 are given in Table 35.

TABLE 35

Body, Ovarian and Adrenal Weights at 45 Days of Age  
(Weanling Experiment II)

Diet	Avg body wt (g)	Ovarian wt (mg) <sup>a</sup>	Ovary (% body wt)	Adrenal wt (mg) <sup>a</sup>	Adrenal (% body wt)
1	25.9	8.3 ± .47	.032	6.5 ± .38	.025
Cf42	23.7	7.4 ± .32	.031	7.1 ± .42	.030
Cf43	23.0	6.7 ± .43	.029	6.8 ± .43	.030
Cf44	21.3	5.8 ± .36	.027	6.5 ± .31	.031
74	24.7	7.3 ± .43	.030	6.0 ± .27	.024

<sup>a</sup> Mean ± SE.

The ovaries in animals fed diet Cf44 weighed significantly less than controls ( $P < .05$ ). Adrenal weights were variable. When expressed as percentage of body weight, adrenal weight was highly correlated with the presence of forage in the diet. Increases in adrenal weights resulted in all cases of forage feeding. A direct correlation was found between ovarian weight at day 45 and growth rate during the log-growth phase (Table 36).

TABLE 36  
Ovarian Weight and Growth Rate  
(Weanling Experiment II)

Diet	Growth rate <sup>a</sup> (g/day)	Avg Ovarian wt (mg)
1	0.99	8.3
Cf42	0.81	7.4
74	0.79	7.3
Cf43	0.69	6.7
Cf44	0.67	5.8

<sup>a</sup> During log-growth phase.

(c). Weanling Experiment III. In order to avoid the body weight variation encountered in the first two weanling experiments, animals were selected so that there would be no differences in starting weights. Fifty 18-day-old females, averaging 0.26 g in weight, were assorted into five equal weight groups of ten animals each. Controls were fed diet 1, and experimental groups were maintained on diets Cf45, Cf46, Cf47 and 74. The various diets are explained in Tables 9 and 11. The procedure for feeding, weighing, and smearing was as described in Weanling Experiments I and II. The feeding schedule is shown in Table 37. Vaginal patency at stage 5 was determined according to the procedure of Hartman, Littrell and Tom (1946). All animals were sacrificed at 45 days of age and, at this time, freshly extirpated ovaries and thymus glands were weighed.

TABLE 37  
Feeding Schedule  
(Weanling Experiment III)

Age	g Diet/mouse	
	8 AM feeding	6 PM feeding
18-19 days	---	2.0
20-27 days	1.0	2.5
28-34 days	1.0	3.0
35-45 days	1.0	4.0

Vaginal estrous activity (Table 38) was consistently similar in animals fed diets 1, Cf47 and 74. Estrous index, the number of estruses, and the number of animals showing an estrus by day 45 were greatly reduced in animals receiving diets Cf45 and Cf46. Vaginal introitus and relative growth rate were not significantly different (Table 39).

TABLE 38  
Vaginal Estrous Activity  
(Weanling Experiment III)

Diet	No. Animals	No. Smears	No. Estrous smears	No. Estruses	Estrous index <sup>a</sup>	% Animals showing an estrus by day 45
1	10	174	22	14	13	90
Cf45	10	181	11	8	6	70
Cf46	10	173	13	6	7	50
Cf47	10	183	22	15	12	90
74	10	168	23	14	14	80

$$^a \text{ Index} = \frac{\text{No. Estrous smears}}{\text{No. Smears}} \times 100.$$

TABLE 39  
Vaginal Opening and Relative Growth Rate (K)  
(Weanling Experiment III)

Diet	Vaginal introitus <sup>a</sup> (days of age)	K <sup>b</sup>
1	27 $\pm$ 1.0	.029
Cf45	26 $\pm$ 0.9	.027
Cf46	27 $\pm$ 1.1	.025
Cf47	26 $\pm$ 0.8	.026
74	27 $\pm$ 1.2	.026

a Mean  $\pm$  SE for stage 5.

b K was computed for log-growth phase.  
See Table 31 for formula.

Body weights were similar throughout the feeding period, and no major differences were apparent. A reduced ovarian weight at day 45 (Table 40) was found in animals maintained on diets Cf45 and Cf46. The fact that diet 74 animals had an average ovarian weight higher than controls (diet 1) can not be explained. Diet Cf46 ovarian weight was considerably below that for controls ( $P = .33$ ). The data in Table 40 show that forage feeding results in increased thymus weight at day 45. Thymus glands from animals fed diets Cf45, Cf46 and Cf47 had consistently higher weights than controls ( $P = .33$ ). Cellulose feeding (diet 74) did not bring about this result. An inverse relationship was found between ovarian weight and thymus weight.

TABLE 40

Body, Ovarian and Thymic Weights at 45 Days of Age  
(Weanling Experiment III)

Diet	Avg body wt (g)	Ovarian wt (mg) <sup>a</sup>	Ovary (% body wt)	Thymus wt (mg) <sup>a</sup>	Thymus (% body wt)
1	23.1	7.4 ± .68	.032	75 ± 4.6	.32
Cf45	22.9	6.8 ± .63	.030	87 ± 6.1	.38
Cf46	21.9	6.0 ± .73	.027	92 ± 8.4	.42
Cf47	23.0	7.2 ± .92	.031	84 ± 3.4	.37
74	23.0	8.4 ± .75	.037	78 ± 3.9	.34

<sup>a</sup> Mean ± SE

(d). Weanling Experiment IV. Forty-eight weanling females, with an average weight of 9.23 g, were selected at 18 days of age. These were divided into six equal weight groups, with eight animals in each. One group was used as controls and fed diet 1. The other groups were fed 20% Ladino clover (diet Cf48) and fractions prepared from a sample of this same material. The fractionation procedure is described in the Materials and Methods section, and the extraction scheme is shown in figure 8. The feeding schedule is explained in Table 41.

The experimental procedure was identical to that used in Weanling Experiment III. In addition, daily feed consumption was estimated by weighing food not eaten during a particular feeding period and subtracting this from the amount offered. Data on average diet consumption, vaginal

patency and relative growth rate (K) are given in Table 42. Figure 13 shows approximate consumption of the various diets throughout the experiment.

TABLE 41  
Feeding Schedule  
(Weanling Experiment IV)

Age	g Diet/mouse	
	8 AM feeding	6 PM feeding
18-19 days	---	2.0
20-27 days	1.0	2.5
28-34 days	1.5	3.0
35-45 days	2.0	4.0

No significant differences in body weights occurred, and final weights were practically identical. Estrous activity could not be evaluated accurately because control animals showed an abnormally low number of estrous smears. Consequently, serious doubt exists as to the validity of results obtained from experimentally fed animals. For this reason, data pertaining to estrous activity, ovarian weights and thymus weights will not be reported.

The results in Table 42 indicate, however, that the final alcoholic extract (diet E71) removed the factor(s) responsible for premature vaginal opening. As measured in this experiment, animals maintained on diet Cf48 and diet E71 exhibited vaginal patencies which occurred significantly sooner than in controls ( $P = .05$ ).

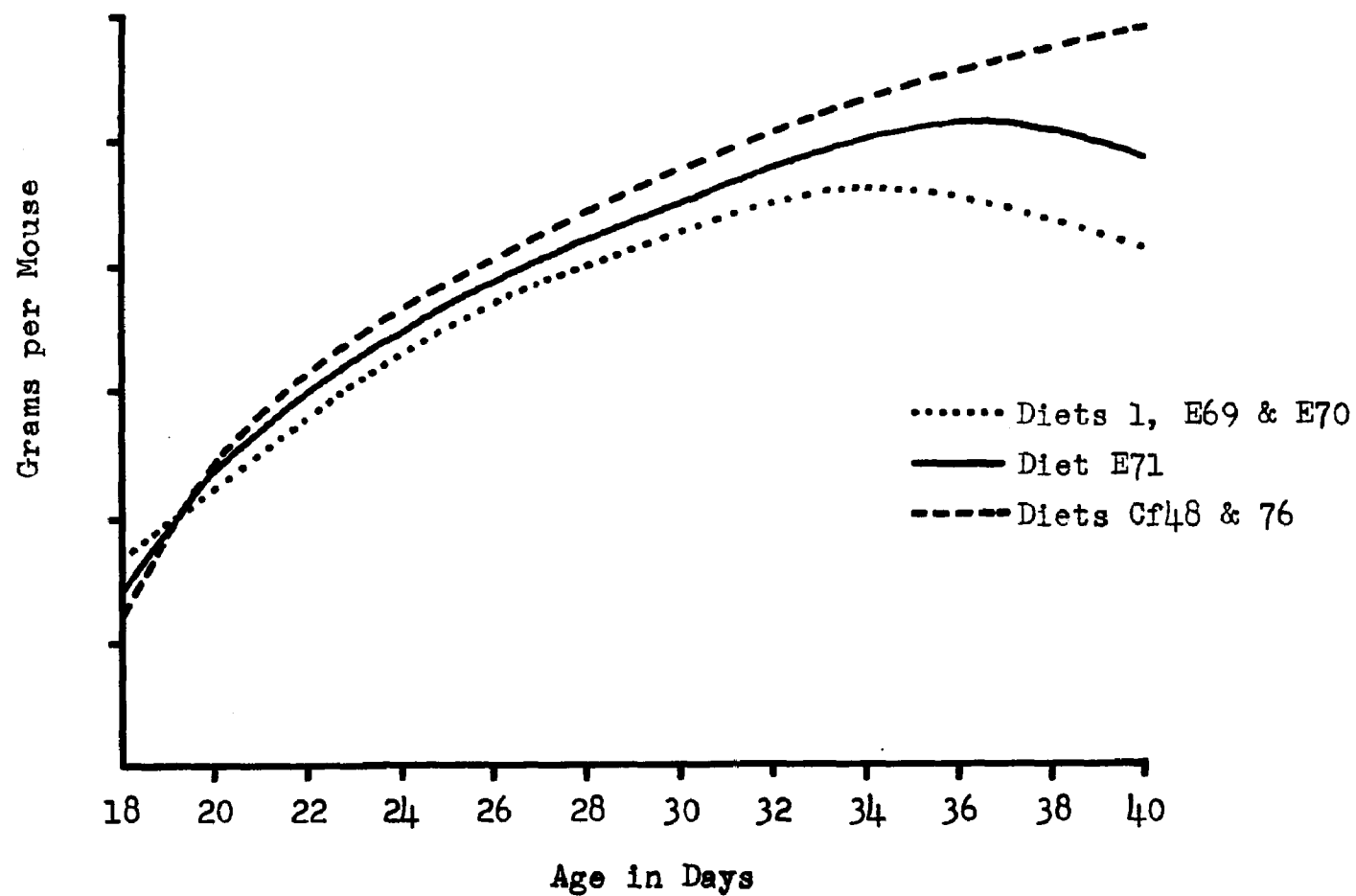
TABLE 42  
 Relative Growth Rate (K), Average Diet Consumption  
 and Vaginal Opening  
 (Weanling Experiment IV)

Diet	K <sup>a</sup>	Avg diet consumption per mouse (g)	Vaginal introitus (days of age) <sup>b</sup>
1	.032	3.76	30 ± 0.7
Cr48	.028	4.11	25 ± 1.1
E69	.032	3.84	28 ± 1.5
E70	.033	3.69	29 ± 1.4
E71	.031	3.99	24 ± 0.9
76	.031	4.12	29 ± 1.2

<sup>a</sup> K was computed for log-growth phase. See Table 31 for formula.

<sup>b</sup> Mean ± SE.

Fig. 13. Estimated Diet Consumption in Weanling Experiment IV.





#### 4. EFFECTS OF DIETS ON THE GROWTH OF OVARIAN TRANSPLANTS

(a). Preliminary. A means of ascertaining the effect on the ovary of protracted stimulation by the pituitary was found by Biskind and Biskind (1944). These authors noted that ovarian tumors resulted after ovaries were transplanted to the spleen. It appeared that the pituitary was freed from the normal inhibitory action of secreted estrogen when the gonad was situated in the hepatic portal system of circulation. Gitsch (1958) implanted ovaries and uteri into the spleen of rats, and observed that the transplanted ovary remained functional in secreting its normal hormonal component. It is well-known that the liver performs a role in estrogen metabolism. The fact that ovarian transplants in the spleen remain functional leads to the assumption that the liver inactivates estrogens secreted by the transplanted ovary, and very little reaches the pituitary in an active form.

This evidence brought forth the notion that bilaterally ovariectomized female mice with ovarian autotransplants in the spleen might be useful test subjects for studying possible influences of dietary factors on the endocrine system. In the normal female, cyclic changes in the reproductive system prove to be too disadvantageous for determinations of specific effects under constant endocrine states.

Moreover, the unpredictable cyclic variations which occur in the estrous cycles of female mice make quantitative work almost impossible.

Preliminary studies were carried out to determine the time necessary for the development of detectible increases in ovarian weight following transplantation to the spleen. Also, the method of transplanting ovaries was investigated in order that reproducible results would be possible with such a technique.

At first, attempts were made to insert ovaries under the splenic capsule. However, it was found that vascularization of the transplant was extremely variable in this position. Many times the ovary became dislodged and was found free in the peritoneal cavity or attached elsewhere in the abdomen.

The technique finally decided upon was as follows:

1. The animal was anesthetized with Nembutal. Two milligrams per 30 g body weight was injected intraperitoneally.
2. Both ovaries were removed through a dorsal incision. The tips of the uteri were ligated prior to removal of the ovaries.
3. The spleen was then exposed through the dorsal incision.
4. A hole was punctured near the caudal pole of the spleen with a trocar. The trocar was 2.5 mm in diameter, and its tip was sharpened to a cutting edge.
5. One ovary was removed from its capsule under a dissect-

ing microscope. It was then inserted into the cavity previously prepared in the spleen.

6. The implanted ovary was held in position until blood released by the splenic puncture clotted around it.
7. Two interrupted sutures were used to close the incision in the peritoneal wall, and two metal wound clips were sufficient for the apposition suture.

Four CD-1 females four months old were prepared using this technique. Vaginal smears revealed that cornified cells did not reappear subsequent to the third day following the operation. After a postoperative period of seven days, the vagina and vaginal smears had reverted to the anestrus condition. At day 14, the animals were sacrificed and the transplants were examined. The ovarian implants could be seen from either side of the spleen and large follicles were obvious. The four ovaries removed at the time of transplantation (day 1) had an average weight of 8.1 mg. The four transplanted ovaries at day 14 averaged 14.5 mg. It was concluded that a 14-day postoperative period allowed detectable ovarian hypertrophy.

(b). Transplant Experiment. Forty adult CD-1 females were checked for estrous activity by means of vaginal smears. Each was bilaterally castrated, and one of the ovaries was implanted in the spleen using the technique outlined in the previous subsection. The transplantation operation was carried out at times when females exhibited cornified vaginal smears. Most transplants were made during late proestrus or estrus.

On the day of transplantation, animals were started on various dietary regimens. Five were considered negative controls and fed diet 1. Diets 75, Cf49, E72 and E73 were fed to respective groups of four animals each. These diets are recorded in Tables 8, 9 and 11. Positive controls (four animals) were maintained on diet 78. This latter diet contained diethylstilbestrol (DES) at levels alleged to interfere with ovulation (Austin and Bruce, 1956). Vaginal smears were taken daily on each animal during the 14-day feeding period. All diets were fed each day at 2 g per mouse in the morning and 4 g per mouse in the evening.

At the end of the feeding period, animals were sacrificed by cervical separation. The pituitary was immediately removed from the depression on the sphenoid bone and fixed in Zenker's fluid for four hours. Ovarian implants were excised intact under a dissecting microscope and placed in Bouin's fluid for 24 hours. Animals showing adhesions between the transplant and the body wall were discounted from the results. Uteri were extirpated, trimmed of adhering connective tissue, and weighed in the fresh condition.

Serial sections at 5 micra were prepared from the pituitary glands. Heidenhain's Azan procedure (Gray, 1954) was used for staining acidophils in the anterior pituitary. The Mallory-Heidenhain technique as modified by Rona and Morvay (1956) was used in attempts to differentiate basophils. Ovarian implants were serially sectioned at 15 micra and stained with eosin and Ehrlich's acid alum hematoxylin (Gray, 1954).

The histology of pituitaries was difficult to interpret because differential counts of specific cell types were impossible. The staining techniques employed did not allow differentiation of LTH-acidophils nor identification of gonadotrophs as distinct from thyrotrophs. Therefore, little can be said concerning the comparative effects of the diets on the finer details of pituitary cytology. A general increase in the size of the pituitary was noted in negative control animals bearing ovarian autotransplants. Much of the enlargement could be attributed to increased numbers of basophils.

The Trichome-PAS procedure of Pearse (1950) was used to compare pituitaries from negative controls and from normal intact females. It was felt that this procedure might allow FSH-producing cells to be distinguished. Pituitaries from intact CD-1 adult females were fixed in Helly's fluid for four hours and sectioned at 5 micra. Comparable sections taken through anterior lobes are shown in figures 14, 15 and 16. In transplant animals many of the peripheral basophils had larger, more irregular nuclei. This may indicate the hypersecretory state of these cells.

Ovarian transplants in negative control animals showed many enlarged follicles. The diameters of these ranged from 400 to 1100 micra. The modal type had a diameter of about 600 micra. Measurements, however, were found to be variable, since many enlarged follicles were not completely ovoid. In implants containing 10 to 15 enlarged follicles, many elongate, distorted structures were observed. These

probably resulted from compression by adjacent follicles.

The degree of ovarian hypertrophy in animals receiving diets 75, Cf49, E72, and E73 was judged according to structural relationships with either negative controls (diet 1) or positive controls (diet 78). Three general categories were then established. Complete hypertrophy was considered applicable to negative controls, all of which showed ovarian stimulation in the form of large follicles. Intermediate hypertrophy was applied to groups of animals which showed a variable degree of ovarian stimulation. Negative hypertrophy was considered characteristic of positive controls. In this case no follicles were observed beyond the stage of antrum formation (diameter = 350 micra). The results of hypertrophy evaluation and uterine weight determination are given in Table 43.

TABLE 43

## Characteristics of Ovarian Implants and Uteri

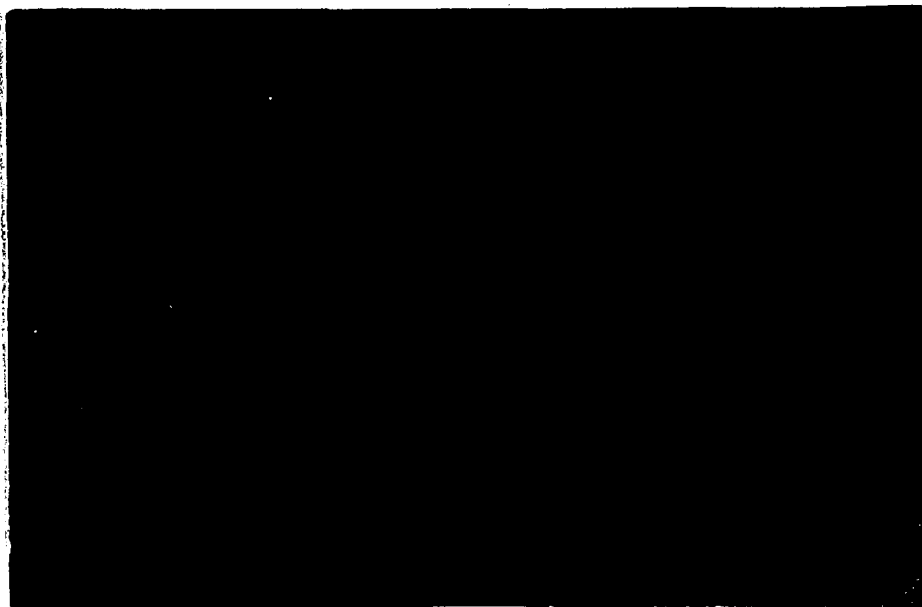
Diet	No. Animals	Degree of ovarian hypertrophy	Uterine wt <sup>a</sup> (mg)
1 <sup>b</sup>	5	Complete	56 $\pm$ 3.3
75	4	Intermediate	53 $\pm$ 3.9
Cf49	4	Intermediate	59 $\pm$ 1.3
E72	4	Negative	76 $\pm$ 21.2
E73	4	Complete	54 $\pm$ 5.6
78 <sup>c</sup>	4	Negative	189 $\pm$ 6.2

<sup>a</sup> Mean  $\pm$  SE

<sup>b</sup> Negative control

<sup>c</sup> Positive control

Fig. 14. Normal Pituitary from Female CD-1  
(1000x)

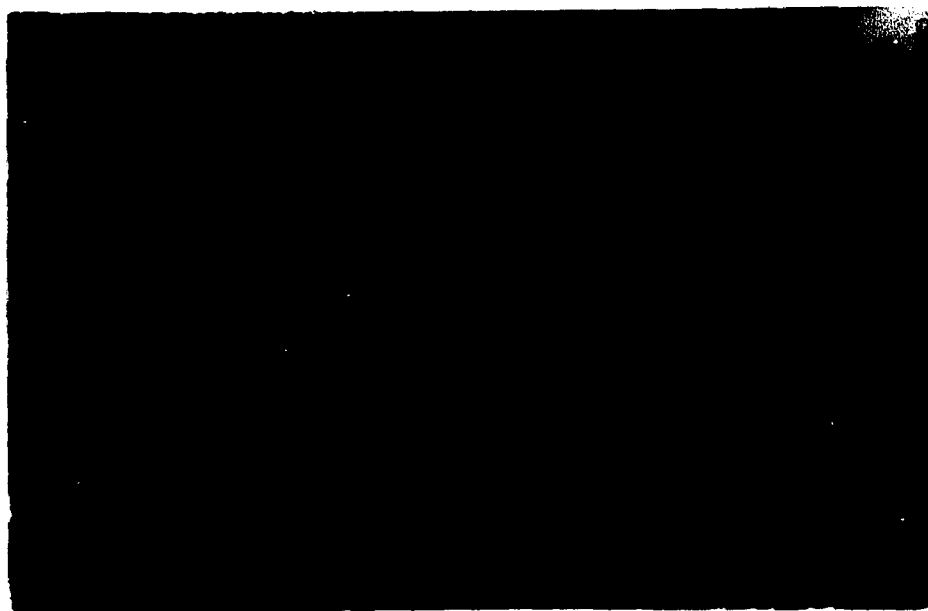


Helly's fixed and sectioned at 5 micra.

Stained with Trichrome - PAS (Pearse, 1950).

Shows a clump of peripheral basophils (pink cytoplasm).

Fig. 15. Pituitary From Negative-control,  
Transplant Animal (1000x)



Zenker's fixed and sectioned at 5 micra.

Stained with Trichrome - PAS (Pearse, 1950).

Shows peripheral basophils, several of which  
are enlarged with irregular nuclei.



Fig. 16. Pituitary From Negative-control,  
Transplant Animal (1000x)



Zenker's fixed and sectioned at 5 micra.

Stained with Mallory-Heidenhain stain as modified  
by Rona and Morvay (1956).

Shows basophils with dense cytoplasmic granulation  
(aniline blue positive).

Ovarian hypertrophy more closely resembled negative controls in animals receiving diets 75, Cf49, and E73. The uterine weights of these animals also were similar to negative controls. No stimulation of transplanted ovaries was observed in animals fed an acetone extract (diet E72) of the Ladino clover used in diet Cf49. Uterine weights of these animals were extremely variable. High uterine weights were associated with luteinization of the transplant in animals receiving diet E72.

Animals treated with diet E73 consumed much less food material than animals on the other diets. Actual measurements were not made, but considerable dietary refuse was observed throughout experimental feeding. Therefore, the results obtained from these animals are not actually analogous to the others.

Photomicrographs of typical ovarian implants from animals treated with the diets in question are shown in figures 18 through 23. Figure 17 is a comparable section from a luteinized ovary removed on the day of transplantation.

Fig. 17. Typical Ovary on Day of Transplantation



Fig. 18. Negative Control Transplant.  
(Diet 1)

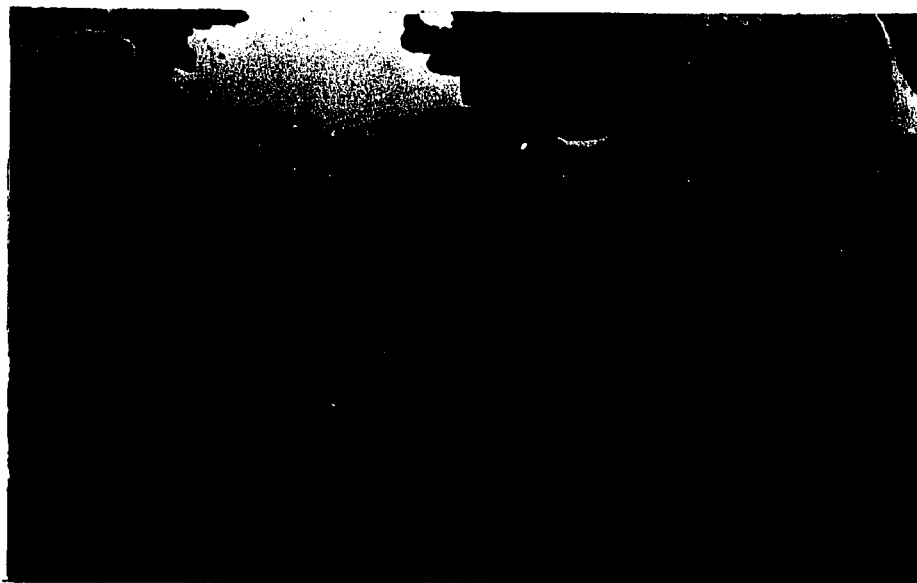


Fig. 19. Positive Control Transplant  
(Diet 78)

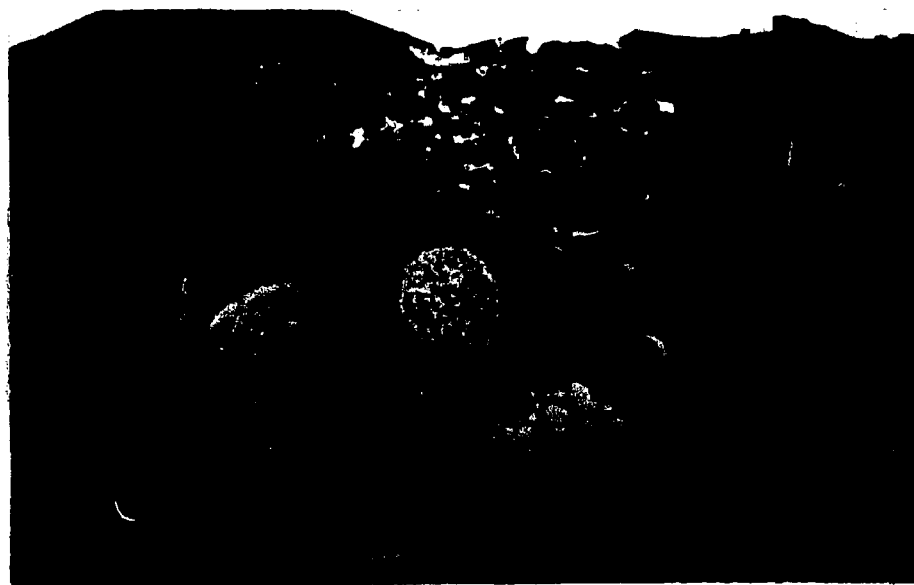


Fig. 20. Transplant From Animal Fed 30% Cellulose  
(Diet 75)

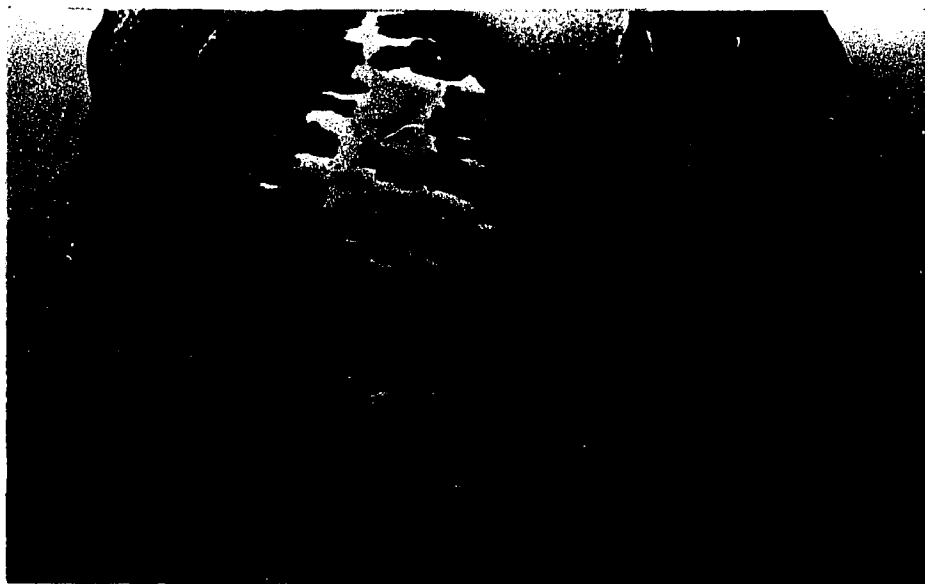


Fig. 21. Transplant From Animal Fed 30% Ladino  
(Diet Cf49)



Fig. 22. Transplant From Animal Fed Acetone  
Extract (Diet E72)



Fig. 23. Transplant From Animal Fed Chloroform  
Extract (Diet E73)



## 5. OTHER STUDIES

(a). Vaginal Smear Experiment I. The pipette or lavage method (Snell, 1941) was used in taking smears. The methylene-blue-stained smear was evaluated in terms of the percentage of nucleated epithelial cells, cornified cells and leukocytes. Daily smears of adult SWR/Jax mice allowed the selection of five females with regular cycles. These were then treated with diet Cf35. This ration was tested in Reproduction Trial I, and the same feeding procedure was used in this experiment. The effect on estrous cycles is shown in figure 17.

(b). Vaginal Smear Experiment II. Twenty adult CD-1 females were selected for estrous activity, by means of vaginal smears. Diet 1 was fed ad libitum to these animals for 16 days, and daily smears were taken throughout this period. Then an experimental feeding and smearing period of the same duration followed. During the latter period, one half of the animals (ten) received diet Cf38 at 6 g per mouse per day. The remaining ten females were fed diet 77 at the same level.

Figure 18 shows characteristic smears for the various stages of the estrous cycle as evaluated in this experiment. These can also be considered as typical stages for other experiments involving the application of vaginal smears.

Fig. 24. Vaginal Cornification in SWR/Jax Mice Fed Diet Cf35

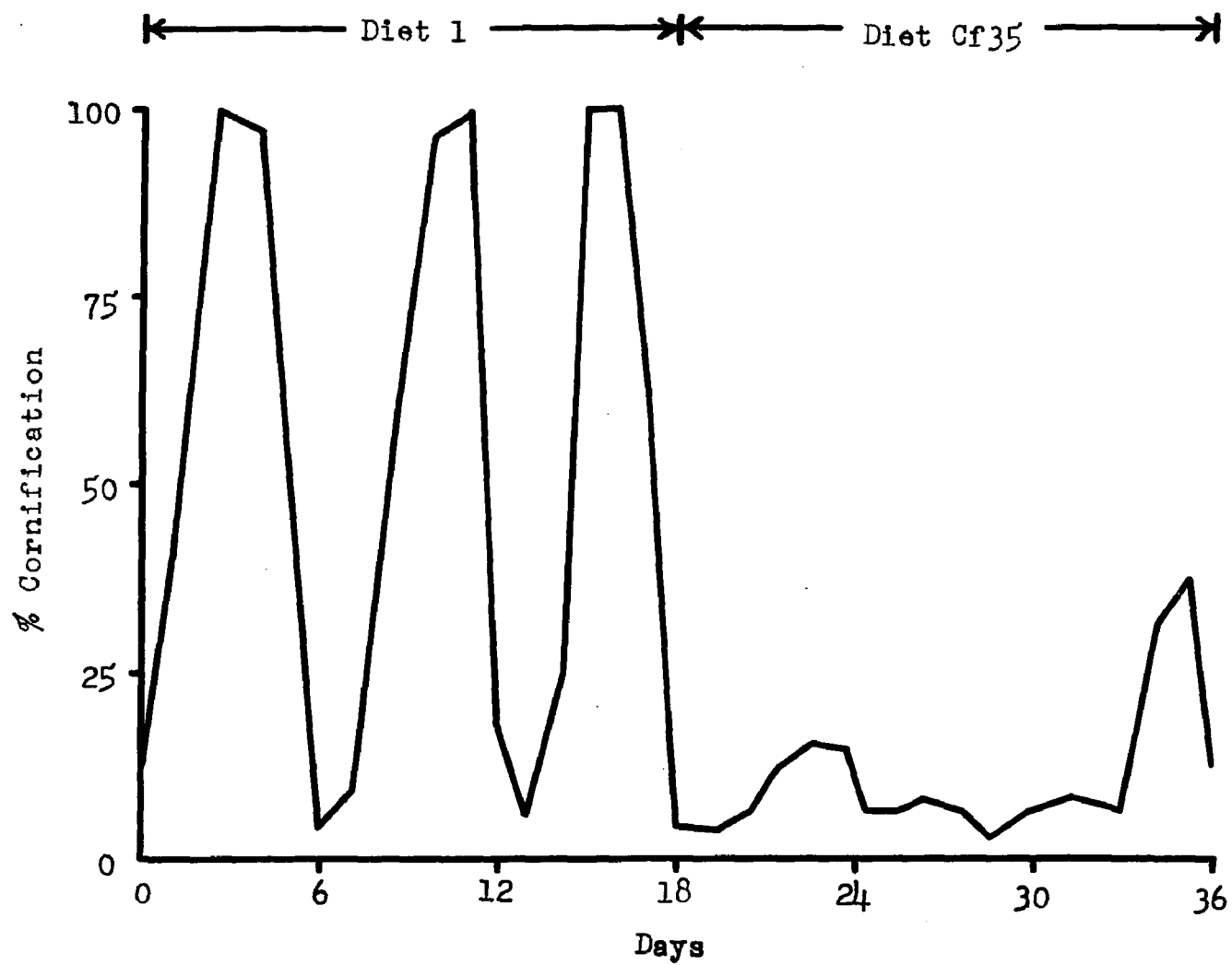
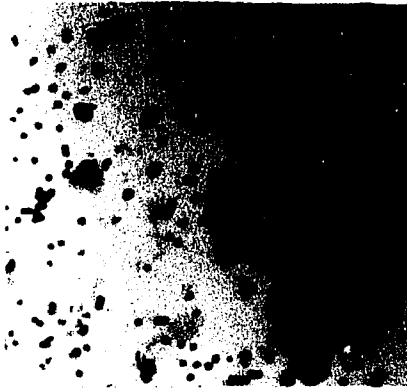


Fig. 25. Characteristic Vaginal Smears For CD-1 Mice  
(100x)

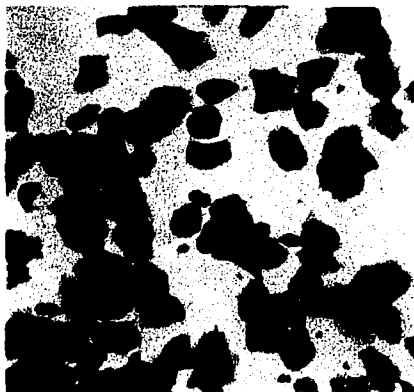
Diestrus - Proestrus (DP)



Late Proestrus (P)



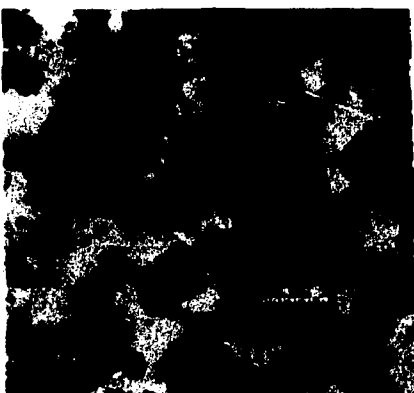
Late Estrus (E)



Metestrus 1 ( $M_1$ )



Metestrus 2 ( $M_2$ )



Diestrus (D)

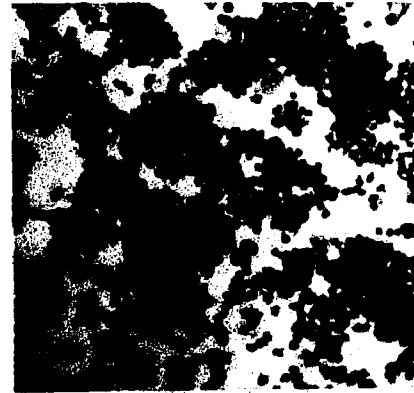




Table 44 shows the results of vaginal smears taken during the control feeding period (diet 1) and during the experimental feeding period (diets 77 and Cf38). An increase in DP smears occurred when diet Cf38 was fed. If stages E,  $M_1$  and  $M_2$  are considered to represent cornified smears, then 35% and 37% of the smears from animals fed diet 1 and diet 77, respectively, were cornified. However, only 22% of the smears were cornified in animals receiving diet Cf38. Therefore, it would seem that Ladino residue (diet 77) does not produce the same effects as whole plant material (diet Cf38). The primary effect of Ladino clover feeding on the estrous cycles of CD-1 mice appears to be an inhibition of vaginal cornification accompanied by an increase in anestrus smears (DP).

TABLE 44  
Vaginal Smear Indices According To Stage<sup>a</sup>  
(Vaginal Smear Experiment II)

Stage	Diet 1	Diet 77	Diet Cf38
DP	0.20	0.19	0.32
P	0.23	0.29	0.29
E	0.18	0.12	0.08
$M_1$	0.06	0.06	0.08
$M_2$	0.11	0.19	0.06
D	0.22	0.15	0.17

<sup>a</sup> Index = Number of smears of a stage divided by the total number of smears in a treatment.

(c). Ova Collection Experiment. The object of this study was to test the effects of dietary Ladino clover on ovulation, fertilization and implantation in CD-1 mice. Eighteen adult controls were fed diet 1. Ten controls were subjected to egg counts performed 34 to 37 hours post coitum in the manner described by Lewis and Wright (1935). The remaining controls were positively mated and allowed to litter if pregnant.

Three experimental procedures were devised:

Treatment 1: Six females were mated and fed diet Cf35 for five days.

Treatment 2: Seven females were started on diet Cf35 at early proestrus and mating trials attempted at estrus. Five were sacrificed for egg counts, and two were examined for pregnancy 18 days after mating.

Treatment 3: Twenty females were fed diet Cf35, and mating trials attempted 11 to 22 days later. Egg counts were made on 13 of these, and seven were left intact for pregnancy determination.

Mating behavior and fertility of matings is depicted in Table 45. The results of egg counts are given in Table 46. Ova were collected 34 to 37 hours post coitum in animals which mated. Non-receptive females were sacrificed for egg counts 35 hours after mating trials had been attempted. Most abnormal

eggs were of the fragmented type as described by Lewis and Wright (1935). Other forms of degenerate ova were also considered abnormal. Ova showing obvious signs of mechanical injury, such as a rupture zona pellucida, were not counted in the data shown in Table 46. Two-cell ova were thought to be fertilized since the second polar body was usually present.

TABLE 45

Mating Record and Pregnancies  
(Ova Collection Experiment)

	No. Animals	Matings per trial	% Fertile matings
Control	8	0.89	100
Treatment 1	6	0.85	83
Treatment 2	2	0.67	0
Treatment 3	7	0.48	80

TABLE 46

## Ova Count Data

	One cell egg	Two cell egg	Abnormal egg	No. Animals	No. Eggs
Positively Mated Females					
Control	9%	88%	3%	8	56
Treatment 2	9%	53%	38%	4	49
Treatment 3	26%	66%	8%	6	47
Non-Receptive Females					
Control	94%	0	6%	2	17
Treatment 2	0	0	100%	1	10
Treatment 3	38%	0	62%	7 <sup>a</sup>	32

<sup>a</sup> Three animals had not ovulated.

Two main effects were shown to result from Ladino feeding. First, mating behavior was reduced in animals fed diet Cf35. Second, the percentage of fertilized eggs (two-cell) was decreased as a result of Ladino treatment. Temporary feeding (Treatment 2) caused an increase in abnormal ova and a concomitant decrease in fertilized eggs. More prolonged feeding (Treatment 3) did not produce as many abnormal eggs, but fewer fertilized eggs were recovered.

(d). Interaction Experiment I. Sixty female CD-1 mice, eight to nine weeks of age, were divided into 12 groups of five mice each. These were then spayed according to the procedure given by Emmens (1950). A 21-day postoperative recovery period was allowed prior to treatment with rations. Vaginal smears were used before and during experimental feeding to verify complete ovariectomy and to observe vaginal response to experimental treatment.

The diets used are explained in figure 4 of the Materials and Methods section. Diethylstilbestrol (DES) was added at two levels to portions of each of the diets (Table 47).

Five mice per cage were fed the various rations for five days at 5 g per mouse per day. At the conclusion of this period, the animals were sacrificed, and uteri were excised, blotted, and weighed. Body weights were recorded prior to and at the conclusion of experimental feeding. Uterine weights according to treatment group are listed in Table 48.

TABLE 47  
Treatment Groups<sup>a</sup>  
(Interaction Experiment I)

Diet	Levels of DES		
	None (X)	0.004 $\mu\text{g/g}$ diet (Y)	0.04 $\mu\text{g/g}$ diet (Z)
1 (1)	X1	Y1	Z1
E51 (51)	X51	Y51	Z51
E52 (52)	X52	Y52	Z52
E53 (53)	X53	Y53	Z53

<sup>a</sup> Five animals per group.

TABLE 48  
Uterine Weights (mg)<sup>a</sup>  
(Interaction Experiment I)

Diet treatment	DES Treatment		
	X	Y	Z
1	21.8 $\pm$ 2.0	25.8 $\pm$ 1.4	32.1 $\pm$ 3.3
51	19.6 $\pm$ 0.6	18.3 $\pm$ 1.0	28.7 $\pm$ 2.9
52	23.3 $\pm$ 4.1	17.9 $\pm$ 1.8	35.4 $\pm$ 4.1
53	19.2 $\pm$ 1.0	18.2 $\pm$ 1.2	32.7 $\pm$ 3.6

<sup>a</sup> Mean  $\pm$  SE; five animals per group.

The highest level of DES treatment (Z) produced a highly significant increase in uterine weight ( $P = .01$ ), and cornification of the vaginal epithelium. Treatment Y1

brought about a uterine weight increase when compared to treatment X1. However, DES treatment Y resulted in a decrease below DES treatment X in diet treatments 51, 52 and 53. This latter effect was not significant, however. Body weight changes during treatment were slight, with a mean weight change of not more than one gram (2.5%). Orally administered extracts of Ladino clover did not have a significant effect on uterine weight, and no significant interaction between extractives and exogenous DES could be deduced from this experiment.

(e). Interaction Experiment II. The erratic uterine responses observed in the bioassay results with intact CD-1 immature females suggested the possibility of interaction between exogenous estrogen and endogenous estrogen. To test this hypothesis, exogenous DES and coumestrol, and endogenous estradiol benzoate were administered in various combinations (Table 49). The levels of DES and/or coumestrol in diets 79, 80 and 81 are given in Table 11 of the Materials and Methods section.

TABLE 49  
Treatment Group  
(Interaction Experiment II)

Diet	Level of estradiol	
	None (X)	0.05 $\mu$ g (Y)
1 - Control (1)	X1	Y1
79 - DES (D)	XD	YD
80 - Coumestrol (C)	XC	YC
81 - DES and Coumestrol (DC)	XDC	--

All diets were fed to ovariectomized 19-day-old CD-1 mice. The same procedure was employed as was used in bioassay studies with these animals. Estradiol benzoate, when used, was injected subcutaneously in 0.5 cc corn oil on day 19. Vaginal patency and uterine weight data are provided in Table 50.

The results shown in Table 50 are only preliminary in nature. Coumestrol (treatment XC) was more effective than DES (treatment XD) in producing vaginal patency. Coumestrol and DES together (treatment XDC) produced a purely additive effect on both uterine and vaginal responses. Treatment YD produced additive effects also. Treatment YC, however, resulted in a uterine response above what would be expected from addition of the individual responses of coumestrol and estradiol. This may indicate an augmentation of coumestrol action on the uterus by estradiol or vice versa.

TABLE 50

## Results of Interaction Experiment II

Treatment	No. Animals	Estradiol injection <sup>a</sup> ( $\mu$ g)	Uterine wt <sup>b</sup> (mg)	Avg Body wt (g)	Uterus (% body wt)	Vaginal patency
X1	12	--	7.9 $\pm$ 0.4	16.5	0.05	0%
XD	12	--	12.9 $\pm$ 0.6	16.9	0.08	0%
XC	12	--	11.7 $\pm$ 0.6	16.9	0.07	92% <sup>c</sup>
XDC	9	--	16.9 $\pm$ 0.8	16.7	0.10	100%
Y1	6	0.05	19.3 $\pm$ 0.8	16.9	0.11	100%
YD	6	0.05	24.0 $\pm$ 1.1	16.8	0.14	100%
YC	5	0.05	25.6 $\pm$ 1.2	17.0	0.15	100%
Diet 1	12	0.00	8.4 $\pm$ 0.3	16.9	0.05	0%
Diet 1	6	0.1	24.5 $\pm$ 1.1	16.0	0.15	100%
Diet 1	5	0.5	57.7 $\pm$ 2.7	17.4	0.33	100%

<sup>a</sup> Estradiol benzoate injected in 0.5 cc corn oil on day 19.

<sup>b</sup> Mean  $\pm$  SE

<sup>c</sup> All were weak responses (stage 4).



## V. DISCUSSION

### 1. ESTROGEN BIOASSAY

The data from bioassays reported in the Experimental Procedure and Results section indicated that low levels of estrogens were usually present in most Ladino clover samples. A few Ladino samples contained enough activity to produce significant responses in uterine weight. Most alfalfa samples appeared to contain slightly higher levels of activity. Samples 24 and 25, supplied by a dairy farmer with a herd experiencing breeding problems when fed this material, were found to have very high estrogenic activity. Two strains of mice were used for forage estrogen bioassay, and various procedures were employed in an attempt to obtain consistent results with control animals.

The biological estimation of estrogenic activity in legume forage has been attempted by numerous investigators, and reference has been made to some of these works in the Review of the Literature and the Materials and Methods sections. Different workers using different methods have found widely divergent levels in the materials tested.

Uterine weight increase has been used commonly to detect estrogens in extracts from legumes when administered by injection in oily solution. Robinson (1949) used spayed or intact immature mice of an unspecified inbred strain, and

injected ether extracts suspended in peanut oil for three days. Cheng et al. (1953) administered "purified" extracts in olive oil for four days to immature intact mice of the C.F.W. strain. Pope, McNaughton and Jones (1959) employed immature mice from a colony in Compton, England, and injected for three days ether and aqueous extracts dissolved in either aqueous propylene glycol or arachis oil. Chury (1960), using ovariectomized rats and immature mice, injected purified alcoholic extracts for four days.

Administration per orum and detection of uterine response has been another common method used in forage estrogen bioassay. Pieterse and Andrews (1956) fed alcoholic extracts for ten days to 10- to 12-week-old ovariectomized mice of the Rockland Swiss strain. Bickoff et al. (1959) fed ether or acetone extracts to 19- to 21-day-old female mice of the Dalswiss strain. Kitts et al. (1959) fed alcoholic extracts over a three day period to intact 21-day-old mice of the U.B.C. Swiss Albino strain.

Kallela and Vasenius (1962) fed ether extracts by means of a stomach tube to mature-spayed albino rats, and estrogenic response was determined according to the Allen-Doisy method, by evaluating the degree of vaginal cornification. Munford and Flux (1961) fed, for six days, diets in which 20% freeze-dried legume had been incorporated. Response was measured by both uterine weight increase and vaginal opening in immature ovariectomized mice.

The preceding summarization of bioassay procedures

used by various workers well exemplifies the lack of standardization of methods in this area of research. Therefore, in lieu of discussing the bioassay methods used in this study and contrasting the results with those obtained in other studies, evidence from this dissertation will be compared with other data to show the need for newer and more standardized procedures for the biological estimation of estrogenic activity in plant material.

Uteri from ovariectomized mice used as controls are relatively small. Robinson (1949) reported for Bouin's fixed uteri from mice, approximately 30 days old, a value of 5.6 to 7.1 mg. Munford and Flux (1961) gave values of 5.6 to 6.0 mg for uteri from mice ovariectomized at 21 to 23 days of age. The results reported in this dissertation showed that fresh uteri from immature ovariectomized CD-1 mice weighed 6.2 to 7.8 mg. All of these data seem to be in good agreement.

Uteri from intact immature mice used as controls do not have identical weights. Chury (1961) reported control uteri from immature mice to weigh 3.8 mg after preserving in alcohol-formalin and blotting before weighing. The U.B.C. strain, as reported by Kitts et al. (1959), have uterine weights of 16.7 to 20.5 mg at 22 to 24 days of age. Cheng et al. (1953) gave control uterine weights of 7.7 mg for immature mice of the C.F.W. strain. This value was determined after fixation in Bouin's fluid and blotting between filter papers. Bickoff et al. (1959) found fresh control uteri from 25- to 29-day-old mice of the Dalswiss strain to

weigh  $9.73 \pm 0.33$  mg (mean  $\pm$  SE). In the present study, Bouin's fixed uteri from SWR/Jax mice weighed  $27.1 \pm 0.9$  mg at 35 days of age. Fresh uterine weights from control intact immature CD-1 mice provided the following: 30 days old =  $26.9 \pm 6.4$  mg, 27 days old =  $30.0 \pm 5.4$  mg, 23-25 days =  $20.8 \pm 2.3$  mg.

The differences observed in the preceding data are partially explained by the different treatments given to the uteri prior to weighing. However, major incompatibilities still exist between the uterine weights of immature intact mice. It would seem that this observation can be largely attributed to differences in the rates of maturation of the various mouse stocks.

Engle and Rosasco (1927) found with "albino" mice that the age at vaginal opening ranged from 28 to 49 days with the median at 35 days. Snell (1941) recognized that the age at vaginal introitus varies considerably both within and among stocks. This author summarized data on the mean age at first estrus. He noted that in an albino strain, this occurred at 39 days of age, whereas in a colored strain the mean age was 52 days. These results clearly indicate that the age at which maturity occurs in different strains is by no means comparable. Moreover, Drasher (1955) found marked strain differences in the early response of the mouse uterus to estrogens. It does not seem unwarranted to assume that such differences exist in the various mouse strains used for plant estrogen bioassay.

Harris (1961) stated that the functional activity of the ovary and anterior pituitary in the immature animal does

not depend on an intrinsic property of the tissue, but rather on the environs in which it is located. In the prepuberal condition, the anterior pituitary and gonads are functionally active, but their activity is usually low. In view of this fact and the great genetic differences occurring between inbred strains, the considerable genetic variation inherent in heterozygous strains, and the many external factors known to affect the gonadal-hypothalamo-pituitary axis, the bioassay results obtained with different stocks of mice cannot be collectively evaluated with any degree of accuracy. Adoption of a single strain of test animal together with rigid standardization of procedures is absolutely necessary.

A comparison between immature intact, ovariectomized, and hypophysectomized mice revealed that more reliable bioassay results were obtained when immature mice were ovariectomized and subsequently tested. In addition to uterine weights, the uterus as percentage of body weight was recorded so that the effect of different final body weights would be minimized. Also, vaginal patency was determined to give additional evidence of estrogenic stimulation. A continuation of this procedure by other workers might help in the interpretation of forage estrogen determinations.

In most cases, uterine weight increase has been selected as the criterion for estrogen determinations because it provides quantitative data. It is generally assumed that most estrogens will create a response at very low doses. However, Biggers (1959) concluded that, in the biological

estimation of estrogenic activity in plant material with mice, vaginal cornification was more suitable than uterine weight response, it being reasoned that cornification, unlike uterine weight increase, was a specific reaction to estrogens.

From results in Tables 20 and 50, data on the relative potencies of DES and coumestrol can be calculated. DES at 0.015  $\mu$ g per gram of diet produced about the same uterine response as coumestrol at 60  $\mu$ g per gram of diet. DES, therefore, would be 4000 times more potent than coumestrol in this respect. DES at 0.03  $\mu$ g per gram diet was about as effective as coumestrol at 60  $\mu$ g per gram diet in causing vaginal patency. In this case, DES is only 2000 times more potent than coumestrol.

Bickoff et al. (1959) calculated the relative potencies of DES and coumestrol and found that DES was 4000 times more potent than coumestrol when measured by uterine weight increase. More recently, Bickoff et al. (1962) compared the relative potency of forage estrogens, DES and estrone by means of the amount necessary to produce a 25 mg uterine weight. DES, in this case, was found to be 3000 times more potent than coumestrol, and at least 100,000 times more potent than genistein and related isoflavones. Data presented by Munford and Flux (1961) showed that DES was about 83,000 times more potent than genistein as an inducer of uterine growth. Other data from these same authors suggested that DES was only 20,000 times more potent than genistein in causing vaginal patency in immature ovariectomized mice after six days of treatment.

Hisaw et al. (1962) tested genistin and coumestrol

acetate for their ability to stimulate uterine growth and fluid imbibition by the uterus according to the Astwood method. Genistin was found to be a poor promoter of both uterine growth and fluid imbibition. Coumestrol acetate stimulated a small amount of uterine fluid imbibition. DES was 1400 times more potent in growth-promoting properties than coumestrol acetate, and estradiol was 51 times more potent in this respect.

The most important feature of these results on the relative potencies of plant estrogens would seem to be that the immature mouse uterus responds to these compounds to a far lesser degree than does the vaginal membrane. This difference in spectrum of activity has received little attention from other workers, even though bioassay of forages using this response would obviously be more sensitive than uterine weight increase. Another advantage of such a procedure would be that test animals need not be sacrificed at the end of the bioassay period. Quantitative determinations would be more difficult with this method, but when used in conjunction with uterine response, more reliable results would be obtained.

Perhaps it is not too much to hope that, in the future, the use of bioassays may become obsolete and that refined and sensitive chemical measurements will replace them. The chromatographic methods used by Guggolz, Livingston and Bickoff (1961) and by Chury (1961) are steps in this direction.

## 2. REPRODUCTION STUDIES

The results of Reproduction Trial I with SWR/Jax mice were comparable to those reported by Fox et al. (1957). These authors fed 40% red clover diets to mice and observed that no conceptions resulted during treatment. Fertility was restored when control diets were substituted for red clover rations. Vaginal Smear Experiment I showed that estrous smears did not occur in SWR/Jax females treated with a 30% Ladino diet for a period of the same duration as the mating period in Reproduction Trial I. This observation could well explain the infertility brought about by Ladino feeding.

Reproduction Trials II and III with CD-1 mice demonstrated that male fertility was not seriously reduced by Ladino feeding. Females did not reproduce normally when treated with a 30% Ladino clover diet. Some females did eventually mate, but mating behavior was curtailed. Vaginal Smear Experiment II showed that Ladino feeding resulted in an inhibition of vaginal cornification and an increase in the anestrus condition. Acetone-extracted Ladino did not produce all of these effects to the same extent.

The Ova Collection Experiment revealed that many abnormal or degenerate eggs resulted when Ladino diets were first offered. After prolonged feeding, the percentage of abnormal ova was reduced to normal levels, but the number of fertilized eggs collected was still below the value for controls. Also,



mating behavior of females was reduced by Ladino treatment, and non-receptive individuals frequently did not ovulate.

It is interesting to compare these results with the findings of Sanger and Bell (1960). These authors studied ova collected from mated ewes. One group of ewes had been grazed on Ladino clover and the other on bluegrass. Of the ova collected from Ladino-fed animals, only 59% were fertilized, whereas 75% of those recovered from bluegrass-grazed animals were fertilized. A reduction of 16% in the number of recoverable fertilized ova was then attributed to an uncharacterized effect of Ladino clover. In Table 46, a reduction of 22% in the number of two-cell ova can be seen to result from prolonged Ladino treatment.

Comparison of the degree of infertility brought about by Ladino feeding with two different mouse stocks suggested that the SWR/Jax strain was affected more severely than was the CD-1 strain. Drasher and Zahl (1946) found strain differences in the sensitivity of mice to the effects of dietary Lithospermum. Although the inhibitor in this plant material may have physiological properties dissimilar to the effects of Ladino, it appears that some sort of analogy exists. Drasher and Zahl (1946) found that C<sub>3</sub>H mice went into immediate and persistent anestrus when treated, while the Rockland strain developed varying degrees of refractoriness to treatment. Recently, Breneman et al. (1960) extracted the causal agents from Lithospermum, but noted that inanition was probably a complicating factor when the whole plant material

was incorporated in the diet of mice.

Inanition may be an important contributing factor when crude forage diets are fed to mice. In Reproduction Trial I weight losses occurred upon initial treatment with Ladino, and the amount of weight reduction was related to the percentage of forage in the diet. The females began to regain weight after 18 days of treatment, indicating adaptation to the diets. CD-1 females seemed to be affected less harshly in this respect.

Dilution of the diet does not explain completely the weight losses upon initial treatment. The growth data from the Weanling Experiments indicated that diets diluted with cellulose bring about some reduction in growth rate. Certain crude forage diets also caused growth rates as low as, or lower than the growth rates of animals fed cellulose diets. However, mice on other crude forage rations had growth rates equal to the rate for controls.

Diet consumption was measured in Weanling Experiment IV, and it was shown that animals receiving crude forage or extracted residue in the diet consistently ingested more than animals fed control ration or experimental extract diets. Digestibility of two 30% Ladino diets in adult female CD-1 mice was determined, and the results are shown in the Appendix. The digestibility of fat, fiber, protein, and nitrogen-free extract was found to decrease when either vegetative or second-growth clover diets were fed.

In view of these results, a tentative hypothesis can be proposed to explain some effects of Ladino feeding on mice.

When animals are initially treated with crude forage rations, dilution of the diet is a major factor and a certain amount of nutritional stress is placed on the organism in that diet consumption at normal levels does not supply nutrients in sufficiently digestible quantities. The animals then compensate for this imbalance by increasing their consumption of the forage diets.

The reason for the lower digestibility of clover diets would appear to be related to their higher fiber content. However, Conrad et al. (1958) found only half of carbon-14 label in the feces of rats after administration of uniformly-labeled carbon-14 soybean cellulose in the diet. The rest of the label was found in expired  $\text{CO}_2$ , carcass and urine. Bacterial action was presumed to be responsible for the digestion of the cellulose. These findings suggest that similar results could be anticipated with the mouse. Consequently, much of the cell wall portion of Ladino clover would be expected to be digested, cell contents being freed for assimilation. If this assumption is valid, initial effects of Ladino diets can be attributed to dietary dilution. However, after the digestive tract has adapted to the ration, digestibility of forage diets should increase. Since this was not the case, it would seem that other factors are involved in prolonged feeding of Ladino. An alternative explanation of the data does not seem justified at the present time.

In Extract Reproduction Experiments, the incorporation of Ladino clover and alfalfa fractions in the diet of CD-1

females did not produce effects peculiar to unfractionated Ladino. Estrous smears did not revert to the anestrus condition and most females readily mated. Fertility of matings was generally high and normal litters were cast.

Swierstra (1958) fed alcoholic extracts of dried red clover and birdsfoot trefoil to mice and noted impaired reproductive performance. He attributed these results to the presence of estrogen-like substances in the extract diets. Females were more severely affected than males. Two different red clover extracts produced the same degree of reduced fertility, but there was a three-fold difference in estrogenic activity of these diets. A birdsfoot trefoil preparation had an estrogenic activity at least ten times less than either of the red clover diets, but produced more infertility. Three animals died as a result of feeding the trefoil extract diet. Significant reduction in diet consumption occurred with all extract diets and body weights decreased as a result of treatment.

Forage estrogens may explain some of the results reported by Swierstra (1958). However, in view of an unsubstantial correlation between estrogenic content of the diet and the degree of infertility produced, it would seem that other factors were certainly responsible for much of the impaired reproductive performance found by this author. Toxicity or unpalatability of the extract diets certainly could have been responsible for some degree of inanition, and this in turn could have produced lower fecundity. Also, the fact that

females were more sensitive to treatment leads to the assumption that the presence of forage estrogens in the diets does not explain the results, because administration of pure genistein (East, 1955d) or coumestrol (Booth, personal communication, 1961) affects male fertility more harshly.

In Extract Reproduction Experiment I fresh Ladino clover with a low estrogenic content was extracted with alcohol and acetone, and these extracts were further purified and tested. No extract adversely affected litter size. The aqueous-acetone extract reduced the willingness to mate and this could be attributed to the fact that treated females did not immediately accept males at first estrus. The opposite situation occurred with aqueous-alcoholic extract. Females receiving this preparation were more willing to accept males than controls.

In Extract Reproduction Experiment II, aqueous-alcoholic extract of Ladino produced a high mating trial record, comparable to results for this fraction in Experiment I. Also, females treated with this fraction accepted males at first estrus more often than did controls. An acetone extract of alfalfa, shown to be estrogenic, produced mating results similar to the aqueous-alcoholic extracts of Ladino. No significant effect on litter size was found with any of the extracts tested in Experiment II.

When the results of Extract Reproduction Experiments I and II are compared with the findings of Swierstra (1958), no correlation between the levels of extracted estrogen and

impaired reproductive performance of female mice can be found. This conclusion obviously does not apply to situations involving ingestion of large amounts of forage estrogen, however. The fertility of sheep grazing highly estrogenic legume pastures is known to be seriously hindered (Moule, 1961). Bovine sterility in this connection is also thought to result. Adler and Trainin (1960) showed that reproductive disturbances in Israel were associated with estrogens in alfalfa hay. The symptoms observed were high incidence of cystic ovaries, nymphomania, too short estrous cycles, abnormally long heat periods, anestrus, and abortions.

In relating reproduction tests with laboratory animals to actual situations in the field, consideration should be given to the fact that the original estrogenic activity may be altered while preparing samples, whether in drying, storing, or while extracting. Bickoff et al. (1960) discussed the loss of estrogenic activity during dehydration of the plant material and subsequent storage. It was noted that coumestrol may be more susceptible to oxidative degradation than the isoflavones, but that all forage estrogens are unstable under these conditions.

Bickoff et al. (1959) showed that extraction of freshly-harvested legume was likely to remove more activity than extraction of dried samples. The bioassay of diets used in Extract Reproduction Experiment II revealed that each of the fractions of alfalfa contained less estrogenic activity than did a bioassay diet (B15) prepared from this sample. Bioassay

of diet B14 prepared from a fresh alfalfa sample taken from the same source was found to be highly estrogenic. These particular samples were suspected of contributing to bovine sterility with symptoms closely resembling those reported by Adler and Trainin (1960). The breeding problem disappeared the following year and associated with this was a marked decline in estrogenic activity (diet B25). Confirmation that estrogens were directly involved was not possible. Moreover, the results of the Extract Reproduction Experiments suggest that other substances might be complicating factors.

The design of the fractionation scheme used in preparing material for the Extract Reproduction Experiments is discussed in the Materials and Methods section. If estrogens alone were involved, diets E62, E63, E66 and E68 should have produced similar results, depending of course on the relative estrogenic content of the diets. This was not the case, however. Diet E68 had the highest estrogenic activity, and it did not appear to cause adverse effects on the reproductive efficiency of females (except, perhaps, for slightly lower litter sizes and a small increase in the number of resorptions).

Diets E62 and E66, ether extract diets, produced one pseudopregnancy each. This effect was also observed with benzene extract (E64) and chloroform extract (E67), producing one and three pseudopregnancies, respectively. Therefore, direct effects of estrogens were not related to the occurrence of repeat breeders. Moreover, the incidence of pseudopregnan-

cies did not appear to be related to diet consumption. In figure 12, it is shown that the largest drops in consumption occurred with diets E65 and E67. However, pseudopregnancies resulted from treatment with diets E66 and E67.

The effects of diet E67 (chloroform extract) may have been unique in that a highly significant decline in body weight resulted when this diet was fed to bioassay animals (Table 20). The presence of growth-depressing substances was considered, and it appeared equally likely that palatability might be involved. Reduced consumption of another chloroform extract diet (E73) was noted in the Transplant Experiment.

Ostrovsky and Kitts (1962) found that the benzene fractionation of an initial alcoholic extract of dried red clover removed factor(s) influencing estrogenic activity. Also, this fraction appeared to be a major contributor to unpalatability of diets prepared from extracts.

Since more pronounced body weight decreases were observed with young, growing animals, the possibility that "palatability" factors may exert a growth-depressing effect on the organism should not be overlooked. Unpalatability of such rations may be a manifestation of physiological effects produced by the diet, resulting indirectly in reduced diet consumption.

Ferguson, Ashworth and Terry (1949) tested 34 flavonoid compounds on their ability to inhibit smooth muscle activity. Many compounds were found to suppress motility at very low concentrations. Ferguson et al. (1950) identified



tricin, a flavonoid from alfalfa, and found that it inhibited smooth muscle activity. East (1955a) discussed rutin and quercetin and noted that they were not estrogenically active, but that a diet containing 0.1% rutin impaired the fertility of female mice. Shaw and Jackson (1957) studied the in vitro effects of Ladino fractions on the oxygen consumption of diaphragm, the motility of smooth muscle, and acetyl cholinesterase activity. An alcohol-soluble fraction and its water-soluble components inhibited all three systems. It was suggested that triterpenoid saponins might be responsible for these effects. Shaw and Jackson (1959) succeeded in isolating cholinesterase and respiratory inhibitors from alfalfa. Both types of substances were found to reside in an alcoholic extract known to contain saponins. Thompson (1959) presented data on some physiological effects of legume saponins. Feeding alfalfa saponins to chicks caused growth retardation when administered at 0.2 and 0.4% in the diet. Extreme irritating effects on the mucous membrane were observed when legume saponins were injected into the small intestines of rats.

It is conceivable that flavonoid and/or saponin compounds could be incriminated in some of the effects noted in studies with legume extract diets. In Extract Reproduction Experiment II, the number of resorptions increased in all cases of extract feeding. Mating behavior may be related to the effects of such compounds as might be the incidence of pseudopregnancies. A saponin extract of Ladino clover was

tested in Interaction Experiment I, but no significant effects on uterine weight were detected. When 0.004  $\mu$ g DES per gram was added to this and other extracts, a consistent reduction in uterine weight was observed. Continuation of these studies might result in the identification of inhibitors of estrogenic activity.

### 3. COLLATERAL STUDIES

Weanling Experiments I and II showed marked differences in the response of weanling mice to various 15% forage diets. Diet Cf43 was compounded from alfalfa known to be estrogenic. Animals fed this ration had a reduced relative growth rate, a normal estrous index, and a slightly premature vaginal introitus. Timothy grass (diet Cf44) produced significantly later vaginal introitus, reduced growth rate, and high estrous index. Ladino clover diets showed differences which were related to the stage of maturity of samples collected from the same source. Prebloom Ladino (diet Cf39) inhibited growth and produced a very low estrous index. Bloom Ladino depressed growth somewhat, but brought about a normal estrous index. Second-growth Ladino (diets Cf41 and Cf42) did not reduce growth rates and seemed to increase estrous index.

In Weanling Experiment III, various stages of Ladino from another source were tested. Both vegetative and prebloom samples inhibited estrous index. A second-growth sample did not potentiate estrous index. It may be pertinent that the second-growth sample in Experiment III was harvested in June, whereas those samples in Experiments I and II were obtained in August.

The results associated with Ladino feeding indicate

that this plant in its early stages of development contains material detrimental to endogenous estrogenic action. Such effects might result as secondary manifestations of growth retardation. Later in the growing season, Ladino appears to have potentiating effects on endogenous estrogenic activity, not related to increased growth rate. Stages between these two extremes exhibit neither inhibiting nor potentiating properties. It is possible, however, that both factors are present in amounts which would nullify the physiological manifestation of each.

It is interesting to note that Ferguson et al. (1949) found the flavonoid content of alfalfa to be higher earlier in the season and much lower in August. Since many of the flavones have inhibitory physiological properties, inhibitory effects of Ladino may be related to a high content of such compounds. Increased estrous activity could then be a result of stimulatory factors operating in the absence of inhibitory flavonoids.

The estrous and growth trends produced by Ladino can not be completely compared to tissue weights at 45 days of age, because the latter were not determined in Weanling Experiment I. In Experiment II, ovarian weight was directly related to growth rate in general. Adrenal weight showed no such correlation, but larger adrenals were observed in all cases of forage feeding. In Experiment III, lower ovarian weights at day 45 were associated with reduced growth rates for forage feeding. However, cellulose feeding resulted in

a slow growth rate, but did not cause reduced ovarian weight. Thymic hypertrophy paralleled reduction of ovarian weight in animals fed Ladino diets. This effect was not observed when animals were treated with a diet diluted with cellulose.

Since dietary dilution with cellulose did not produce effects similar to forage feeding (i.e., increased adrenal weight and hypertrophy of thymus glands), it can be concluded that forages exert physiological effects on the growth of mice which can not be explained by inadequate dietary intake. Adrenal increase may be related to a "stressful" condition produced by forage diets. Larger thymus weights probably indicate a suppression of certain aging phenomena. Estrous activity and ovarian weight were depressed by feeding early stages of Ladino clover. Further appraisal of Ladino's influence on the growth phenomena of mice must await more elaborate and precise experimentation, however.

A bioassay procedure using bilaterally castrated female mice bearing intrasplenic ovarian transplants was developed to allow an evaluation of forage factors affecting both the pituitary and sex accessories. In the Transplant Experiment, it was shown that 30% Ladino and 30% cellulose in the diet produced slight but similar effects on the growth of ovarian implants. When an acetone extract of Ladino was administered, ovarian hypertrophy was suppressed to the same degree as when diethylstilbestrol was administered. Exogenous DES stimulated uteri and suppressed ovarian activity. The acetone extract caused variable uterine weights, but response

was associated with luteinization of the ovarian implant. Evaluation of pituitary function was not possible with the staining techniques used, so effects at this level could not be characterized.

Fox et al. (1958) compared the effects of oral estradiol and 40% red clover on the reproductive organs of mice. It was found that red clover did not mimic the effects of estradiol. This result led to the conclusion that the effects of red clover could not be explained in terms of exogenous hormonal action or in terms of pituitary stimulation or inhibition. However, since feeding whole legume in the Transplant Experiment induced physiological effects dissimilar to extract administration, it would appear that crude forage diets fed to mice do not produce results which can be considered as representing the true effects of the plant material. A similar conclusion was made in the comparison of Reproduction Trials with Extract Reproduction Experiments.

The Ladino sample used for the preparation of the acetone extract diet in the Transplant Experiment did not contain significant estrogenic activity (diet B17). Yet acetone extract resulted in suppression of ovarian hypertrophy. Since the results were not actually comparable to exogenous estrogen treatment (DES diet), it would seem that its mode of effect was different from estrogenic action.

In the future, a comparison of pure plant estrogens and natural estrogens using transplant animals would allow an appraisal of their relative effects on pituitary function.

It is suggested that the PAS-trichrome staining procedure would be useful in conjunction with determinations of gonadotropin content of anterior pituitary glands. After the relationship of forage estrogens to hypophysial function has been determined, various legume fractions could then be tested. Possibly, inhibitory diets could be evaluated with and without addition of natural or forage estrogens.

## VI. SUMMARY

Oral bioassay of Ladino clover and alfalfa for estrogenic potency revealed that low levels of activity were usually present in the samples tested.

1. Various harvesting procedures did not affect the estrogenic activity of Ladino clover.
2. Bioassay determinations with immature intact CD-1 female mice did not produce consistent results with controls.
3. Ovariectomized 19-day-old mice treated for six days gave better results.
4. Response was evaluated by uterine weight increase, premature vaginal patency and uterus as percentage of body weight.
5. Discrepancies in other bioassay procedures were noted, and it was proposed that results obtained from different mouse strains cannot be compared accurately.
6. The relative potencies of coumestrol and diethylstilbestrol were calculated, and it was shown that coumestrol and genistein were more effective as inducers of vaginal patency than as stimulators of uterine weight increase.

Alfalfa hay suspected of contributing to reproductive disorders in a New Hampshire dairy herd was analyzed.

1. High estrogenic activity was present at the time when breeding problems were encountered.



2. The following season, the estrogenic potency of the alfalfa was much lower, and breeding problems in the herd had disappeared by this time.
3. Confirmation that forage estrogens were involved was not directly possible.

When Ladino clover meal was added to the diet of SWR/Jax mice, no litters were cast.

1. Upon initial treatment, decreases in body weight occurred which were proportional to the amount of clover consumed.
2. Females were found to regain weight after a short period of treatment with 15 and 30% Ladino diets.
3. Females returned to a fertile condition approximately five days following the cessation of Ladino feeding.
4. A study of vaginal smears from SWR/Jax mice similarly treated revealed that the estrous condition was inhibited by a 30% Ladino diet.
5. It was felt that estrous inhibition explained the infertility observed.

The fertility of male and female CD-1 mice receiving 30% Ladino diets was tested.

1. Treated males were not affected to any appreciable degree, and fertile matings occurred when males were allowed access to untreated estrous females.
2. Females receiving a Ladino diet did not have normal estrous cycles.
3. Vaginal smears showed that estruses began to reappear

after 18 days of Ladino feeding.

4. Fertile matings did eventually occur, when treated females were paired with untreated males, but mating behavior was seriously curtailed by Ladino treatment.
5. Acetone-extracted Ladino residue and unfractionated Ladino were tested in a vaginal smear study with female CD-1 mice. The Ladino diet inhibited cornification and increased the number of anestrus smears, but the residue diet did not duplicate these effects.

Various Ladino treatments were given to CD-1 females in an attempt to evaluate effects on ova collected after mating.

1. Mating behavior of females was decreased by all treatments. Willingness to mate was reduced to a greater extent by prolonged Ladino feeding.
2. When Ladino was initially fed, many abnormal and degenerate ova were recovered.
3. More prolonged feeding did not produce increases in abnormal ova, but fewer were found to be fertilized.
4. Faulty ovulation was observed in some females treated for 11 to 22 days.

Ladino extracts, when administered in the ration of CD-1 females, did not duplicate the effects produced by treatment with unfractionated Ladino meal.

1. Estrus was not seriously inhibited by any of the extracts tested.
2. Females receiving aqueous-acetone and ether-alcohol extracts showed reduced mating behavior.

3. Mating enhancement was observed in animals treated with aqueous-alcohol extracts.
4. No significant effect on litter size resulted from administration of any extract.
5. Pseudopregnancies occurred in females treated with acetone-ether, alcohol-benzene and alcohol-ether extracts.
6. No pseudopregnancies were observed in females fed control ration or diets containing aqueous extracts.

No correlation was found between infertility and the estrogenic potency of legume.

1. An estrogenic alfalfa sample was fractionated and extracts were tested on CD-1 females.
2. A chloroform extract caused increased resorptions, reduced mating behavior, and pseudopregnancies.
3. An estrogenic acetone-extract increased mating behavior and had no adverse effect on female fertility.

The occurrence of inhibitory flavonoid and saponin compounds in legumes was discussed, and it was suggested that some of these compounds may influence reproductive phenomena. Strain differences were observed in the severity of the infertility produced by Ladino clover diets. The SWR/Jax strain appeared to be more sensitive to the effects of Ladino than the CD-1 strain. Digestibility of 30% Ladino diets was lower than control ration. It was proposed that initial effects of Ladino diets may be a result of nutritional stress, whereas longer feeding results in symptoms which are still

uncharacterized.

Study of the growth of weanling female CD-1 mice fed 15% forage diets disclosed other effects of legumes.

1. Diets diluted with cellulose at 15% by weight caused reduction in growth rate.
2. Many forage diets also caused decreases in growth rate. In some cases the decrease was greater than that induced by cellulose; other times it was much less.
3. Vegetative and prebloom Ladino diets caused reduced growth rate, inhibition of estrous activity as revealed by vaginal smears, and poor ovarian development.
4. Bloom Ladino did not inhibit estrus and ovarian weight increase.
5. Some Ladino samples harvested late in the growing season appeared to enhance estrous activity.
6. Adrenal weights were increased to the same extent by forage diets, but this was not true for a cellulose diet.
7. Thymus glands were larger at 45 days of age in animals receiving vegetative and prebloom Ladino diets. Thymus weight was inversely related to ovarian weight.
8. A cellulose diet did not cause increased thymus weight.

It was concluded that feeding legumes to immature female mice produces effects which cannot be attributed to dilution of the diet. It was suggested that flavonoid substances may be responsible for the inhibitory effects produced by feeding early stages of Ladino clover.

Bilaterally castrated CD-1 mice bearing intrasplenic ovarian autotransplants were fed Ladino clover, extracts prepared from Ladino, cellulose, and diethylstilbestrol.

1. Hypertrophy of the ovarian implant occurred in animals receiving Ladino meal, cellulose, chloroform extract and control ration.
2. Ovarian implants were not hypertrophied when diethylstilbestrol diet and acetone-extract diet were fed. The acetone-extract did not contain detectible estrogenic activity.
3. Since feeding Ladino meal did not simulate results produced by an acetone extract of it, it was suggested that crude forage diets produce side effects which contribute to misinterpretations of real effects.
4. The mechanism of action of Ladino extracts was not characterized, but it was thought to differ from that produced by estrogens.

In general, evidence was supplied that legume per se should not be banished to escape breeding difficulties from its use as forage. These studies indicate that the vegetative stage is likely to be more significant than later stages. Apparently, estrogen-like substances in legumes encountered in local crops are not as important in breeding problems as are other unknown or unidentified estrogen or estrous inhibitors. Forage estrogens are probably more important in the pasture-grazing animal complex. More adequate investigation of this problem is needed, and various suggestions were made which might facilitate work in this direction.

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## APPENDIX

TABLE 51

Digestibility Study<sup>a</sup>

Feeding period <sup>b</sup>	Diet Cf49		Diet Cf50	
	Consumption (g)	Feces (g)	Consumption (g)	Feces (g)
Control	136.5	38.0	132.3	35.4
1	139.2	49.9	135.1	45.6
2	153.5	56.6	137.0	48.0
3	151.9	56.3	140.1	49.8

<sup>a</sup> Eight adult CD-1 females per diet. Results are listed on dry matter basis.

<sup>b</sup> Control period = five days on diet 1. Periods 1, 2, and 3 are successive five-day intervals of experimental feeding.

TABLE 52

Percentage Digestibility of Diet Cf49<sup>a</sup>

Feeding period <sup>b</sup>	Total nutrients	Ash	Fat	Fiber	Protein	N.F.E. <sup>c</sup>
Control	72	37	93	5	66	85
1	64	35	81	2	58	79
2	63	32	80	1	56	79
3	63	32	79	-1	56	79

<sup>a</sup> Results based on dry matter composition.

<sup>b</sup> See Table 51.

<sup>c</sup> N.F.E. = nitrogen free extract.

TABLE 53  
Percentage Digestibility of Diet Cf50<sup>a</sup>

Feeding period <sup>b</sup>	Total nutrients	Ash	Fat	Fiber	Protein	N.F.E. <sup>c</sup>
Control	73	39	93	9	69	85
1	66	40	84	0	63	80
2	65	38	83	-3	58	82
3	65	38	82	-3	57	81

<sup>a</sup> Results based on dry matter composition.

<sup>b</sup> See Table 51.

<sup>c</sup> N.F.E. = nitrogen free extract.